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## Effects of Light Chain Phosphorylation and Skeletal Myosin on the Stability of Non-muscle Myosin Filaments

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The effect of light chain phosphorylation and the presence of skeletal muscle myosin on the stability of non-phosphorylated non-muscle myosin filaments was investigated. Purified skeletal, brush border and thymus myosins were assembled *in vitro* into hybrid filaments consisting of varying proportions of (1) non-muscle and skeletal myosins, or (2) phosphorylated and non-phosphorylated non-muscle myosins. The stability of these hetero- and homopolymers in the presence of MgATP was determined using sedimentation, gel electrophoresis and immunochemical techniques. In addition, the effect of a monoclonal antibody, binding to the tip of brush border myosin tail, on the assembly of the homo- and heteropolymers, was tested. Filamentous non-phosphorylated non-muscle myosin was disassembled by MgATP to the same extent whether in homo- or heteropolymers, indicating that skeletal myosin has no stabilising effect on the hybrid filaments. The presence of small amounts of phosphorylated non-muscle myosin was, however, found to prevent the complete disassembly by MgATP of non-phosphorylated non-muscle myosin filaments, indicating that light chain phosphorylation stabilizes co-operatively non-muscle myosin filaments. The monoclonal antibody prevented the assembly of brush border myosin into both homo- and heteropolymers, and its effect on the filaments was compared with that of MgATP.

### 1. Introduction

Myosins isolated from different species and tissues assemble *in vitro* into filaments with distinct structural features. Vertebrate skeletal muscle myosin filaments are bipolar, ~1.6 μm long, with a small central bare zone, and a homogeneous array of cross-bridge projections (Huxley, 1963; Moos *et al.*, 1975; Hinsen *et al.*, 1978). Vertebrate cytoplasmic myosin filaments are usually bipolar, with a central bare zone, and are 0.3 to 0.6 μm long (Pollard *et al.*, 1978; Hinsen, 1970; Niederman & Pollard, 1975; Mooseker *et al.*, 1978). The molecular basis of the differences observed in the dimensions and shapes of myosin filaments is not yet understood. However, it has been shown that skeletal, smooth muscle and non-muscle myosins can co-assemble into "hybrid" heteropolymers, with morphological features intermediate between those of the respective homopolymers (Pollard, 1975; Kaminer *et al.*, 1976; Wachsberger & Pepe, 1980).

Perhaps the most interesting feature which distinguishes skeletal muscle from smooth muscle/

non-muscle myosin filaments is their stability *in vitro*. Unlike skeletal myosin filaments, the stability of smooth muscle and non-muscle myosin filaments is regulated by light chain phosphorylation under approximately physiological conditions *in vitro*. Skeletal muscle myosin filaments are stable in MgATP. In contrast, non-phosphorylated smooth/non-muscle myosin filaments are disassembled by MgATP, and phosphorylated filaments are stable (Suzuki *et al.*, 1978, 1982; Scholey *et al.*, 1981; Craig *et al.*, 1983; Trybus *et al.*, 1982). In thymus, gizzard and brush border myosins we have proposed that filament assembly/disassembly can be described as a monomer-polymer dynamic equilibrium, regulated by light chain phosphorylation (see Kendrick-Jones *et al.*, 1987).

In the present paper, we investigated the dynamics of filament assembly in non-muscle myosin, and studied how light chain phosphorylation promotes filament formation. Hybrid filaments composed of varying amounts of skeletal and non-muscle myosin, and brush border and thymus myosin homopolymers with different levels of light chain phosphorylation were prepared.

The stability of these filaments in the presence and absence of MgATP and of a monoclonal antibody to brush border myosin was analysed by sedimentation, gel electrophoresis, electron microscopy and immunochemical techniques. The results indicate that, in the presence of MgATP, light chain phosphorylation stabilizes non-muscle myosin filaments in a co-operative fashion, whereas skeletal myosin has no apparent stabilizing influence on the non-muscle myosin present in hybrid filaments.

## 2. Experimental Procedures

### (a) Materials

Fresh bovine thymuses and brains, chicken intestines and gizzards were obtained as described (see Kendrick-Jones *et al.* 1987). Rabbits were obtained from C.A.S., Cambridge, U.K.

### (b) Preparation of proteins

Thymus and brush border myosins were purified, dephosphorylated and thiophosphorylated as described in the previous paper (Kendrick-Jones *et al.*, 1987).

Rabbit skeletal muscle myosin was prepared from combined back and leg muscles by the standard procedure described by Szent-Gyorgyi (1951), with the modifications outlined by Margossian & Lowey (1982). The myosin was further purified by gel filtration on Sepharose CL 4B, using the same conditions as described for thymus and brush border myosins (Scholey *et al.*, 1982; Citi & Kendrick-Jones, 1986), to remove any myosin light chain kinase and any traces of actin or other impurities.

Anti-(brush border myosin) monoclonal antibody BM3 was purified from hybridoma culture supernatant, and monoclonal antibody BM1 was used as ascites fluid (Citi & Kendrick-Jones, 1987).

Myosin light chain kinase and phosphatase (from chicken gizzard) and calmodulin (from bovine brain and thymus) were prepared as described (Kendrick-Jones *et al.*, 1982, 1983).

### (c) Biochemical methods

#### (i) Preparation of myosin filaments and sedimentation assay

All the filaments were prepared by dialysis for 90 min against 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT†, 20 mM-imidazole (pH 7.0). For the heteropolymers, non-phosphorylated non-muscle myosin (brush border or thymus) was mixed at different ratios with skeletal muscle myosin or with phosphorylated non-muscle myosin (all in 1 M-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 25 mM-Tris-HCl (pH 7.5)) so that the final total myosin concentration was ~2 µM, and dialysed. The homopolymers were prepared similarly, except that portions containing varied amounts of each of the non-phosphorylated non-muscle myosin (or skeletal muscle myosin) were dialysed alone. For electron microscopy the filaments were diluted with assembly buffer to a final myosin concentration of 0.6 to 0.7 µM (thymus myosin experiments) or 0.4 to 0.5 µM (brush

border myosin experiments), and immediately negatively stained.

To study the effect of MgATP on the stability of the hetero- or homopolymers, either the dialysis was carried out in the presence of 2.5 mM-ATP, or the nucleotide was added to the filaments after dialysis, with very similar results. After dialysis, the samples (with or without MgATP) were centrifuged at 100,000 g in the airfuge. The myosin concentrations of the sample before centrifugation (*C*<sub>total</sub> = *C*<sub>0</sub>) and of the supernatant after centrifugation (*C*<sub>monomer</sub> = *C*<sub>m</sub>) were determined as described (Kendrick-Jones *et al.*, 1987).

#### (ii) Analytical ultracentrifugation

Analytical ultracentrifugation of skeletal/thymus myosin heteropolymers was performed using a Beckman model E centrifuge, run at 20,000 revs/min, under the conditions described (Kendrick-Jones *et al.*, 1987). Non-phosphorylated thymus myosin and skeletal myosin were mixed in 1 M-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 25 mM-Tris-HCl (pH 7.5), at 1:1 molar ratio, and at a final total concentration of ~4.2 µM. To half of the mixture 2.5 mM-MgATP was added, and then both halves were dialysed separately against 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 20 mM-imidazole (pH 7.0). The apparent sedimentation coefficients (*s*<sub>20,w</sub>) of the monomer species were calculated at this single myosin concentration as described (Kendrick-Jones *et al.*, 1987).

#### (iii) Gel electrophoresis

Glycerol/urea/polyacrylamide gel electrophoresis of the myosin light chains was carried out according to Perrie & Perry (1970). No change was observed in the relative proportions of non-phosphorylated and phosphorylated light chains after non-phosphorylated myosin had been incubated with either skeletal or phosphorylated myosins.

Microslab sodium dodecylsulphate (SDS)/polyacrylamide (5% to 20% (w/v) gradient) gel electrophoresis was performed as described by Matsudaira & Burgess (1978).

#### (iv) Protein concentrations

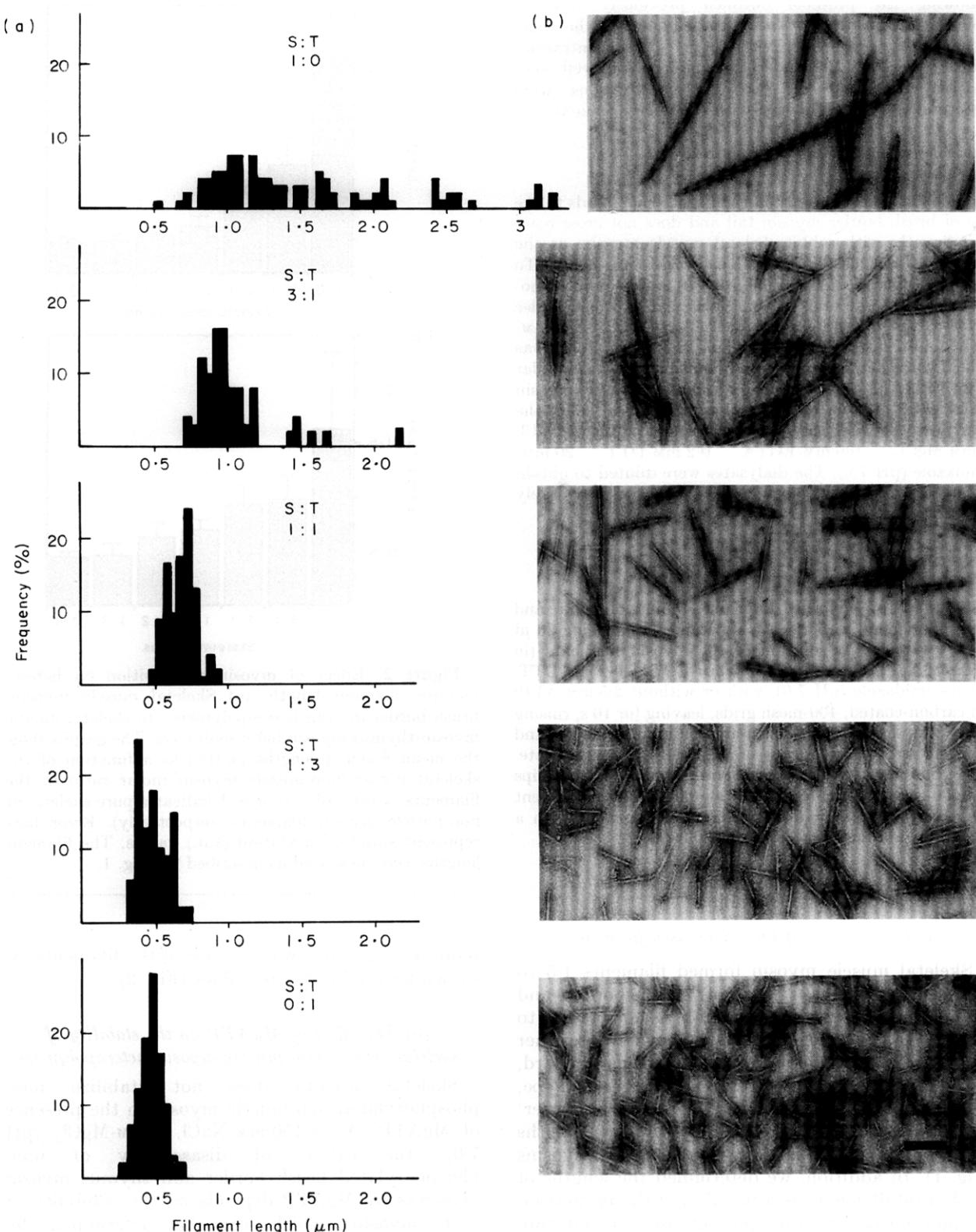
Protein concentrations were determined by the procedure of Lowry *et al.* (1951), or Bradford (1976), using bovine serum albumin as a standard. The concentrations of the myosins were also measured spectrophotometrically, using an absorption coefficient (*A*<sub>280 nm</sub><sup>0.1%</sup>) of 0.54.

### (d) Immunochemical methods

#### (i) Semi-quantitative solid phase assay

A solid phase binding assay was used to measure semi-quantitatively the concentration of brush border myosin in the supernatants after centrifugation in the sedimentation assays. The supernatants were diluted 100-fold in 0.3 M-NaCl, 100 mM-Tris-HCl (pH 8.0), 1 mM-NaN<sub>3</sub>, and 50-µl portions were dried in triplicate in 96-well PVC plates. A reference standard curve was obtained by coating the wells with known amounts of purified brush border myosin, diluted in the same solution. The wells were incubated with first antibody (monoclonal antibody BM1, ascites fluid at a dilution of 1:1000 in PBS containing 1% bovine serum albumin), followed by <sup>125</sup>I-labelled rabbit anti-mouse Ig second antibody.

† Abbreviations used: DTT, dithiothreitol; PBS, phosphate-buffered saline; Ig, immunoglobulin.



**Figure 1.** Thymus myosin/skeletal muscle myosin heteropolymers. (a) Normalized length distributions of skeletal muscle myosin filaments (S) alone (S:T, 1:0), thymus myosin filaments (T) alone (S:T, 0:1), and skeletal/thymus myosin hybrid filaments, prepared by mixing skeletal and thymus myosin at molar ratios of 3:1 (S:T, 3:1), 1:1 (S:T, 1:1) and 1:3 (S:T, 1:3). (b) Representative examples of negatively stained filaments, corresponding to the length distributions shown in (a). The bar represents 0.5  $\mu\text{m}$ . The homo- and heteropolymers were prepared by dialysis against a solution containing 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 20 mM-imidazole (pH 7.0), and negatively stained as described in Experimental Procedures. The lengths of 30 to 100 negatively stained filaments for each specimen were measured on prints at a magnification of 12,000 to 30,000 $\times$ .

following the protocol outlined previously (Citi & Kendrick-Jones, 1987). The cts/min bound to the wells increased almost linearly with the myosin concentration, when between  $\sim 100$  and  $\sim 500$  nmol myosin/well were plated. The unknown myosin concentrations were determined by interpolation with the reference curve.

(ii) *Effect of monoclonal antibody BM3 on filament assembly*

Monoclonal antibody BM3 is an IgM which binds to the tip of brush border myosin tail and does not cross-react significantly with rabbit skeletal muscle myosin, at the concentrations used (Citi & Kendrick-Jones, 1987). To test the effect of this antibody on the assembly of homo- and heteropolymers of non-phosphorylated brush border myosin, the myosin (in 1 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 25 mM-Tris-HCl (pH 7.5)) was mixed with the purified antibody (antibody/myosin molar ratio of 3:1), with or without skeletal muscle myosin (final total myosin concentration of  $\sim 2 \mu\text{M}$ ), and the mixtures were dialysed for 90 min against 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 20 mM-imidazole (pH 7.0). The dialysates were diluted to obtain a myosin concentration of  $\sim 0.4 \mu\text{M}$ , and negatively stained for electron microscopy.

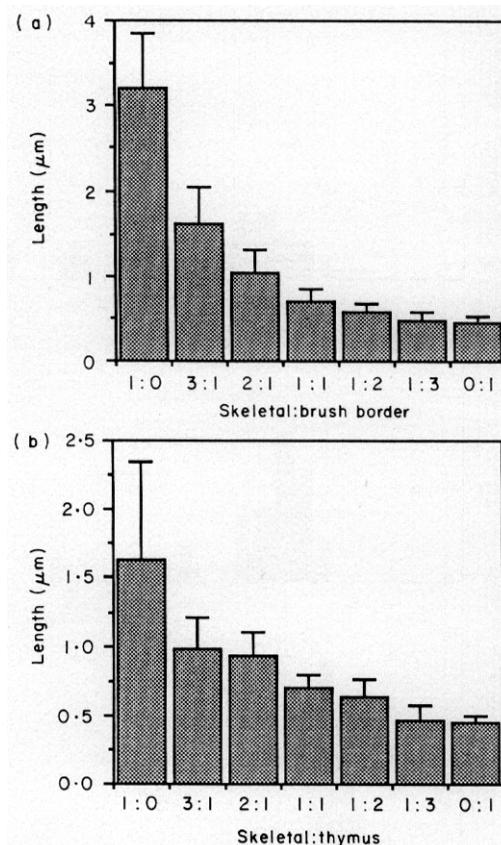
(e) *Electron microscopy*

Samples of myosin filaments (homopolymers and heteropolymers) were negatively stained by placing 20  $\mu\text{l}$  of a 0.4 to 0.7  $\mu\text{M}$ -solution of the myosin filaments (in 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 20 mM-imidazole (pH 7.0), with or without 2.5 mM-ATP) on carbon-coated, 400-mesh grids, leaving for 10 s, rinsing with 12 drops of 240 mM-ammonium formate, and staining with 4 to 8 drops of 1% (w/v) uranyl acetate. The grids were dried in the air and examined in a Philips EM400 microscope, operated at 80 kV. The filament lengths were measured on prints of micrographs with a magnification factor of 12,000 or 30,000  $\times$ .

### 3. Results

(a) *Dimensions of hybrid myosin filaments*

Skeletal muscle myosin formed filaments 1.5 to 3  $\mu\text{m}$  long, whereas those of brush border and thymus myosins were considerably shorter (0.3 to 0.6  $\mu\text{m}$ ) (Fig. 1). As previously shown with other non-muscle and smooth muscle myosins (Pollard, 1975; Kaminer *et al.*, 1976; Wachsberger & Pepe, 1980) heteropolymers formed from brush border/thymus myosin and skeletal myosin had lengths intermediate between those of the pure myosins (Fig. 1). In addition, we determined the lengths of the hybrid filaments as a function of the proportion of non-muscle myosin present in the mixture (Fig. 2). When compared to the pure skeletal muscle myosin filaments, the greatest change in size of the heteropolymers occurred when only 25% of non-muscle myosin was present (skeletal/non-muscle 3:1 molar ratio) (Figs 1 and 2). Thus, the presence of small amounts of non-muscle myosin reduced dramatically the length of skeletal myosin filaments. In addition, the greater the amount of non-muscle myosin present in the mixture, the

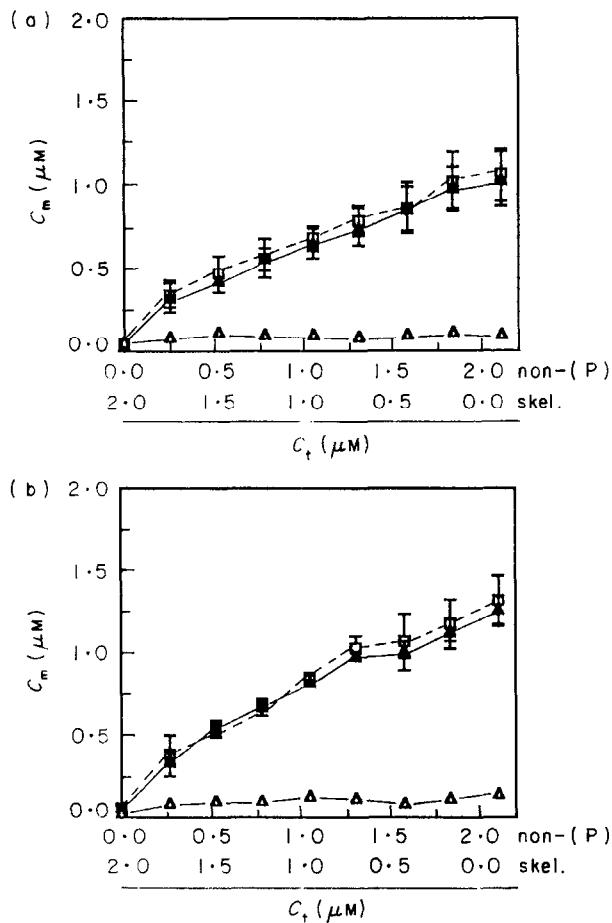


**Figure 2.** Effect of myosin composition on heteropolymer filament length. (a) Skeletal muscle myosin/brush border myosin heteropolymers; (b) skeletal muscle myosin/thymus myosin heteropolymers. The graphs show the mean filament lengths plotted as a function of the skeletal myosin/non-muscle myosin molar ratio in the filaments (a ratio of 1:0 or 0:1 indicates pure skeletal or non-muscle myosin filaments, respectively). Error bars represent standard deviation (S.D.) values. The filament lengths were measured as described for Fig. 1.

more homogeneous was the size of the filaments, as shown by the lower S.D. values (Fig. 2).

(b) *The effect of MgATP on the stability of skeletal muscle/non-muscle myosin heteropolymers*

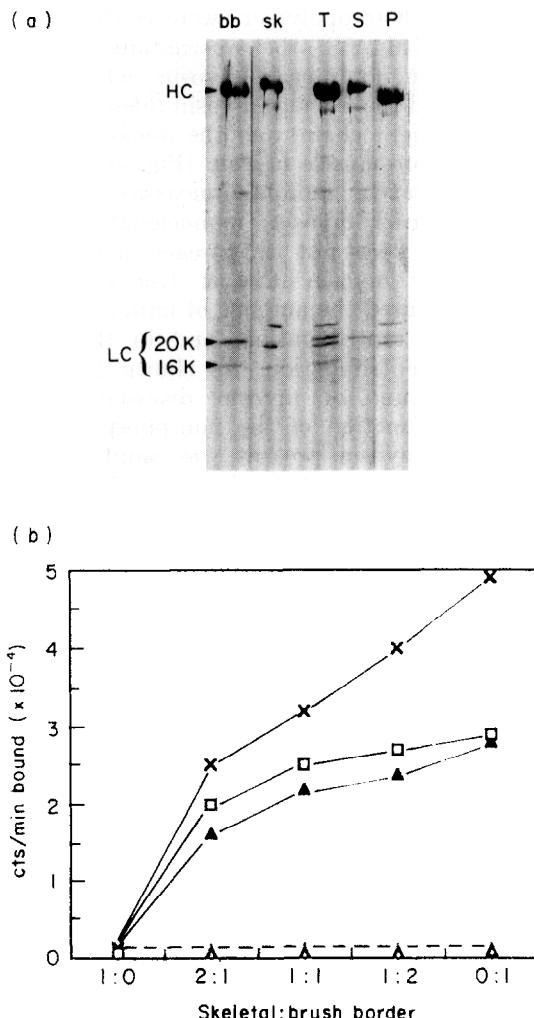
Skeletal myosin does not stabilize non-phosphorylated non-muscle myosin in the presence of MgATP. At  $\sim 150$  mM-NaCl, 5 mM-MgCl<sub>2</sub> (pH 7.0), the extent of disassembly of non-phosphorylated brush border and thymus myosin filaments by MgATP depends on the solubility of each myosin, which can be determined by measuring the concentration of soluble myosin in the supernatant after centrifugation (Citi & Kendrick-Jones, 1986; Kendrick-Jones *et al.*, 1987). When filaments were formed of variable proportions of skeletal and non-muscle myosins, the monomer concentrations in the absence or in the presence of MgATP were very similar to those obtained with the pure non-muscle myosins (Fig. 3), indicating that skeletal myosin had no effect on the



**Figure 3.** Stability of non-phosphorylated non-muscle/skeletal myosin heteropolymers. (a) Plots of monomeric myosin concentration ( $C_m$ ) as a function of the total myosin concentration ( $C_t$ ) for non-phosphorylated (non-(P)) brush border myosin alone and in the presence of MgATP (□), and of mixtures of non-phosphorylated brush border myosin and skeletal (skel.) myosin, in the ratios shown in the graph, in the absence (△) or in the presence (▲) of MgATP. (b) Plots of monomeric myosin concentration ( $C_m$ ) as a function of the total myosin concentration ( $C_t$ ) for non-phosphorylated thymus myosin alone and in the presence of MgATP (□), and of mixtures of non-phosphorylated thymus myosin and skeletal myosin, in the ratios shown in the graph, in the absence (△) or in the presence (▲) of MgATP. The total and monomer concentrations were determined by the sedimentation assay described in Experimental Procedures. Error bars represent standard deviation values, determined on the basis of at least 3 separate experiments. The monomer concentrations for brush border and thymus myosin alone, and in the absence of MgATP, were very similar to those of the respective heteropolymers, and are not shown in the graph for clarity. Note that  $C_t$  for the non-muscle/skeletal myosin mixtures is always  $\sim 2 \mu\text{M}$ , whereas for non-muscle myosin alone it increases from 0 to  $\sim 2 \mu\text{M}$ .

disassembly of the non-muscle myosins. Similar results were obtained whether MgATP was added to the skeletal/non-muscle myosin mixtures before or after dialysis. In the absence of MgATP, the monomer concentrations were always low (Fig. 3).

SDS/polyacrylamide gel electrophoresis showed



**Figure 4.** SDS/polyacrylamide gel electrophoresis and radioimmunoassay analysis of the monomer species. (a) SDS/polyacrylamide (5% to 20% gradient) gel electrophoresis of brush border/skeletal myosin mixtures. bb, brush border myosin alone; sk, skeletal myosin alone (note the extra  $M_r$  25,000 light chain); T, total skeletal/brush border myosin 1:1 mixture, after dialysis and before centrifugation (note the presence of all types of light chains); S, supernatant after centrifugation (note the absence of the  $M_r$  25,000 light chain but the presence of the non-muscle light chain); P, pellet after centrifugation. Equivalent amounts of total, supernatant and pellet were loaded onto the gel. HC, myosin heavy chain; LC, brush border myosin  $M_r$  20,000 (20K) and  $M_r$  16,000 (16K) light chains. (b) Solid phase radioimmunoassay to measure the amount of immunoreactive brush border myosin before and after centrifugation, as a function of filament composition. (x) Skeletal/brush border myosin mixture, before centrifugation; (□) supernatant after centrifugation of brush border myosin alone, in the presence of MgATP; (▲) supernatants after centrifugation of the skeletal/brush border myosin heteropolymers, in the presence of MgATP; (△) supernatants after centrifugation of the skeletal/brush border myosin heteropolymers, in the absence of MgATP. The immunoreactivity of the samples was determined by a 2-step solid phase radioimmunoassay, with a monoclonal antibody to brush border myosin (BM1) followed by a  $^{125}\text{I}$ -labelled second antibody (Citi & Kendrick-Jones, 1987).

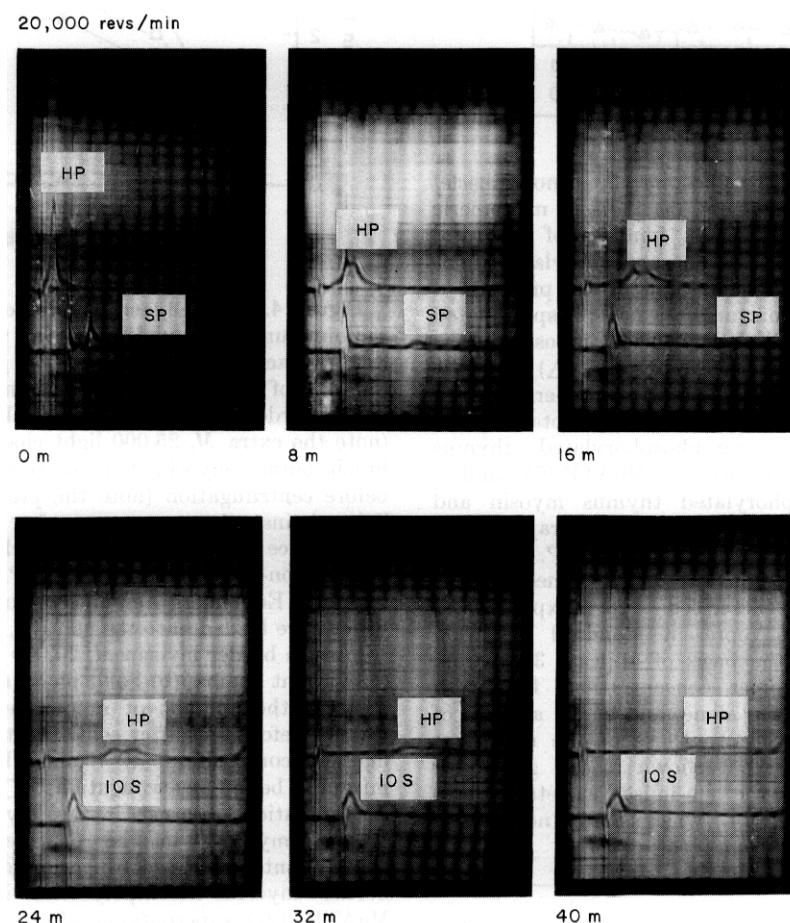
that when the heteropolymers were centrifuged in the presence of MgATP, the supernatant contained essentially only non-muscle myosin, whereas the pellet contained the skeletal myosin (identifiable by its 25,000  $M_r$  light chain) and the remaining, non-disassembled non-muscle myosin (Fig. 4(a)). Using a semi-quantitative radioimmunoassay, with an anti-(brush border myosin) monoclonal antibody (BM1), which does not cross-react with rabbit skeletal muscle myosin (Citi & Kendrick-Jones, 1987), we measured the amount of immunoreactive myosin before centrifugation, and in the supernatant after centrifugation. As shown in Figure 4(b), the amount of myosin disassembled by MgATP as a function of the non-phosphorylated brush border myosin present was similar in the presence or absence of skeletal myosin. This result further confirmed the data shown in Figure 3.

Using the analytical ultracentrifuge we characterized the monomer and polymer species present in skeletal/thymus myosin heteropolymers with or without MgATP (Fig. 5). At equilibrium and without MgATP heteropolymer peaks with  $s_{20,w}$  values in the range of 110 to 125 were

observed (Fig. 5, upper trace). In the presence of MgATP, two well-separated peaks were seen, one rapidly sedimenting ( $s_{20,w}$  value of 285), and one slowly sedimenting, with  $s_{20,w}$  of 10·3 (Fig. 5, lower trace). The value of 285 S is similar to that reported for skeletal myosin filaments (200 to 300 S; Kaminer & Bell, 1966), whereas 10·3 S corresponds to the folded thymus monomer (Craig *et al.*, 1983). Thus, the heteropolymer is disassembled by MgATP into folded thymus myosin molecules (10 S) and polymer, consisting mainly of skeletal myosin.

(c) *The effect of MgATP on the stability of filaments composed of varying amounts of non-phosphorylated and phosphorylated brush border and thymus myosin*

Phosphorylated non-muscle myosin stabilizes non-phosphorylated non-muscle myosin filaments in the presence of MgATP. Filaments of brush border myosin composed of mixtures of non-phosphorylated and phosphorylated molecules were similar in size to those of fully non-phosphorylated

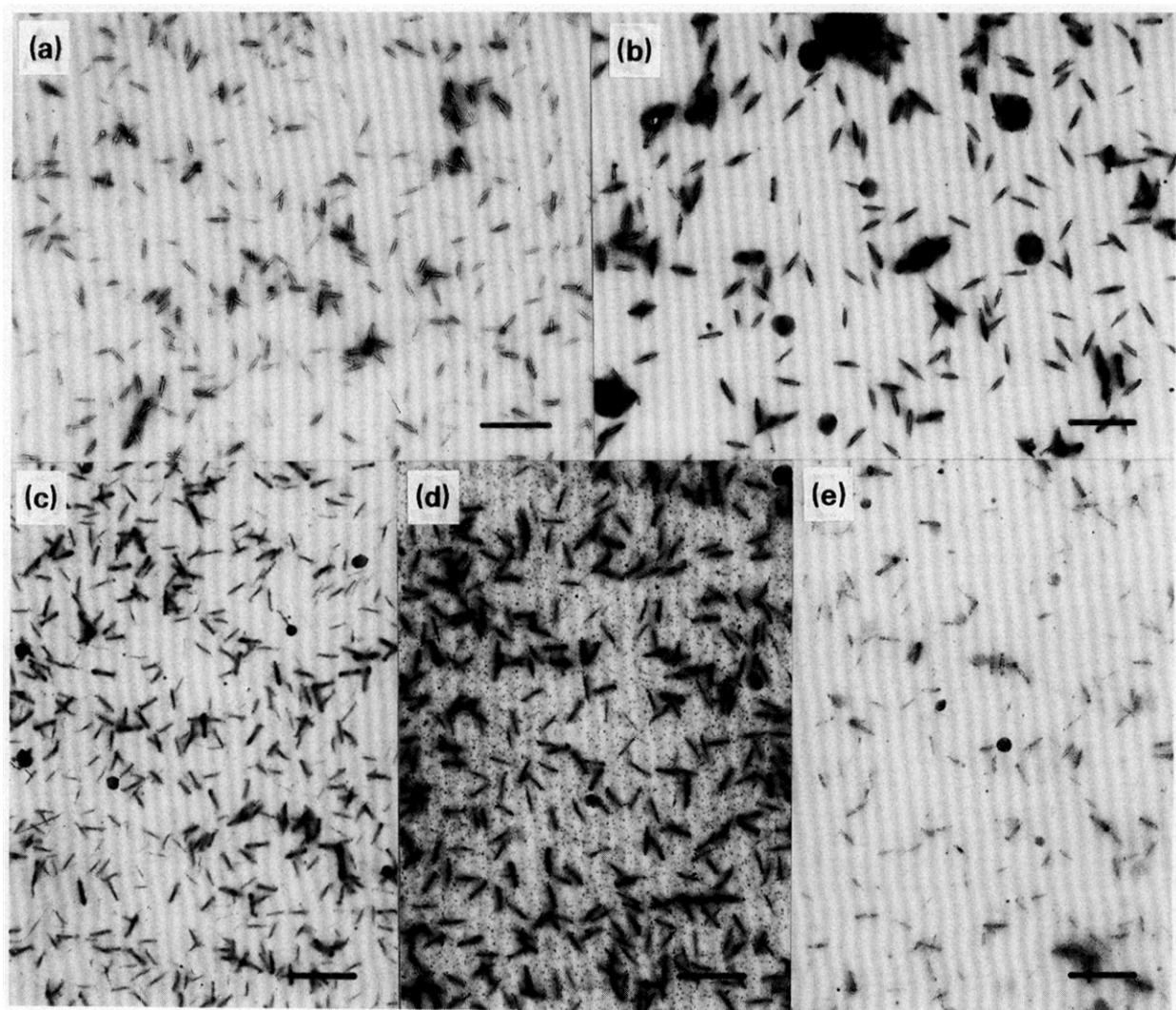


**Figure 5.** Ultracentrifugal analysis of non-phosphorylated thymus/skeletal myosin heteropolymers (1:1 molar ratio) in the presence and absence of MgATP. HP, heteropolymer peak; SP, peak containing mainly skeletal myosin; 10 S, peak corresponding to the folded thymus myosin molecules. The upper trace is the sample without MgATP, and the lower trace that containing 2.5 mM-MgATP (see also Experimental Procedures). The centrifuge was run at 20,000 revs/min, and photographs were taken at the time intervals shown in minutes (m).

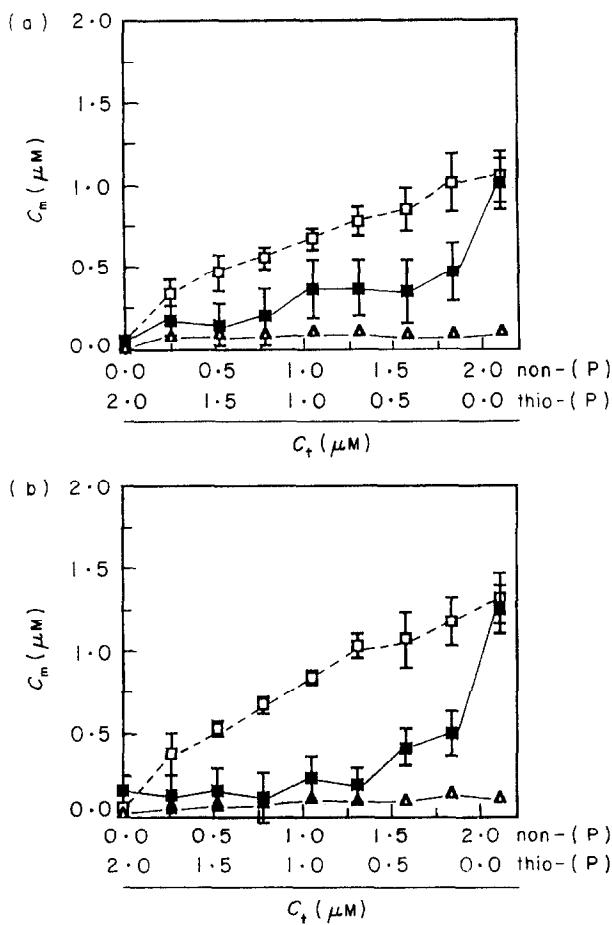
or phosphorylated filaments (Fig. 6: compare (a) and (b) with (d)). In MgATP, small amounts of phosphorylated myosin (25%) protected filaments, composed mainly of non-phosphorylated myosin, from being disassembled (Fig. 6; cf. (d) and (e)). We further analysed this stabilizing effect using the sedimentation assay (Fig. 7). Only when the filaments contained >50% brush border or >75% (thymus) non-phosphorylated myosin were significant amounts of soluble myosin detected in the supernatants (Fig. 7(a) and (b)). In the absence of MgATP, only trace levels of soluble myosin were measured, regardless of the composition of the filaments. The stabilizing effect of phosphorylated myosin was not due to phosphorylation of the non-phosphorylated myosin during the experiment, as shown by glycerol/urea/polyacrylamide gel electrophoresis.

(d) *Effect of monoclonal antibody BM3 on the stability of brush border and skeletal myosin homo- and heteropolymers*

Monoclonal antibody BM3 inhibits completely the assembly of brush border myosin filaments, when added to the myosin at a ~3:1 molar ratio or above (Citi & Kendrick-Jones, unpublished results). The effects of this antibody and MgATP on the homo- and heteropolymers of brush border and skeletal myosin were compared (Fig. 8). Neither MgATP nor the antibody had any significant effect on the mean length and size distribution of the skeletal muscle myosin filaments (Fig. 8(a)). In contrast, non-phosphorylated brush border myosin filaments ( $0.45 \pm 0.06 \mu\text{m}$ ,  $N = 75$ ) were partially disassembled by MgATP (see also



**Figure 6.** Phosphorylated brush border myosin stabilizes non-phosphorylated brush border myosin filaments. Electron micrographs of negatively stained filaments of: (a) phosphorylated brush border myosin filaments; (b) non-phosphorylated brush border myosin filaments; (c) phosphorylated filaments in the present of 2.5 mM-MgATP; (d) filaments containing 25% phosphorylated myosin in 2.5 mM-MgATP (note the relatively low level of disassembly); (e) non-phosphorylated filaments in 2.5 mM-MgATP. The bar represents 1  $\mu\text{m}$ .



**Figure 7.** Stability of non-phosphorylated/non-muscle myosin homopolymers. (a) Plots of monomeric myosin concentration ( $C_m$ ) as a function of the total myosin concentration ( $C_t$ ) for non-phosphorylated brush border myosin alone, in the presence of MgATP (□), and of mixtures of non-phosphorylated brush border myosin and thio-phosphorylated brush border myosin, in the ratios shown in the graph, in the absence (△) or in the presence (■) of MgATP. Note that  $C_t$  for the mixtures is the sum of the non-phosphorylated and phosphorylated myosins and is always  $\sim 2 \mu\text{M}$ , whereas  $C_t$  for non-phosphorylated myosin alone increases from 0 to  $\sim 2 \mu\text{M}$ . (b) Plots of monomeric myosin concentration ( $C_m$ ) as a function of the total myosin concentration ( $C_t$ ) for non-phosphorylated thymus myosin alone, in the presence of MgATP (□), and of mixtures of non-phosphorylated thymus myosin and phosphorylated thymus myosin, in the absence (△) or in the presence (■) of MgATP. The monomer concentrations were determined by the sedimentation assay described in Experimental Procedures. The monomer concentrations for non-phosphorylated brush border and thymus myosin alone, in the absence of MgATP, were very similar to those of the respective mixed polymers, and are not shown in the graph for clarity.

Fig. 3), and the residual filaments were considerably shorter ( $0.26(\pm 0.05) \mu\text{m}$ ,  $N = 80$ ). In the presence of BM3, no brush border myosin filaments were observed (Fig. 8(c)). The skeletal/brush border myosin heteropolymers were homogeneous in size, with a mean length of  $0.69 \mu\text{m}$  ( $\pm 0.14$ ,  $N = 60$ ). On

addition of MgATP, the filaments were clearly heterogeneous in size, with two major populations, with mean lengths of  $\sim 0.25 \mu\text{m}$  and  $\sim 0.75 \mu\text{m}$ , suggesting a mixture of short brush border myosin homopolymers and heteropolymers. In the presence of monoclonal antibody BM3, however, the mean length and the size distribution of the filaments was very similar to that of pure skeletal myosin filaments, indicating that brush border myosin did not co-assemble with skeletal myosin under these conditions, and therefore pure skeletal myosin filaments were formed (Fig. 8(b)). Thus, the net effects of MgATP and the antibody on the heteropolymers were clearly different.

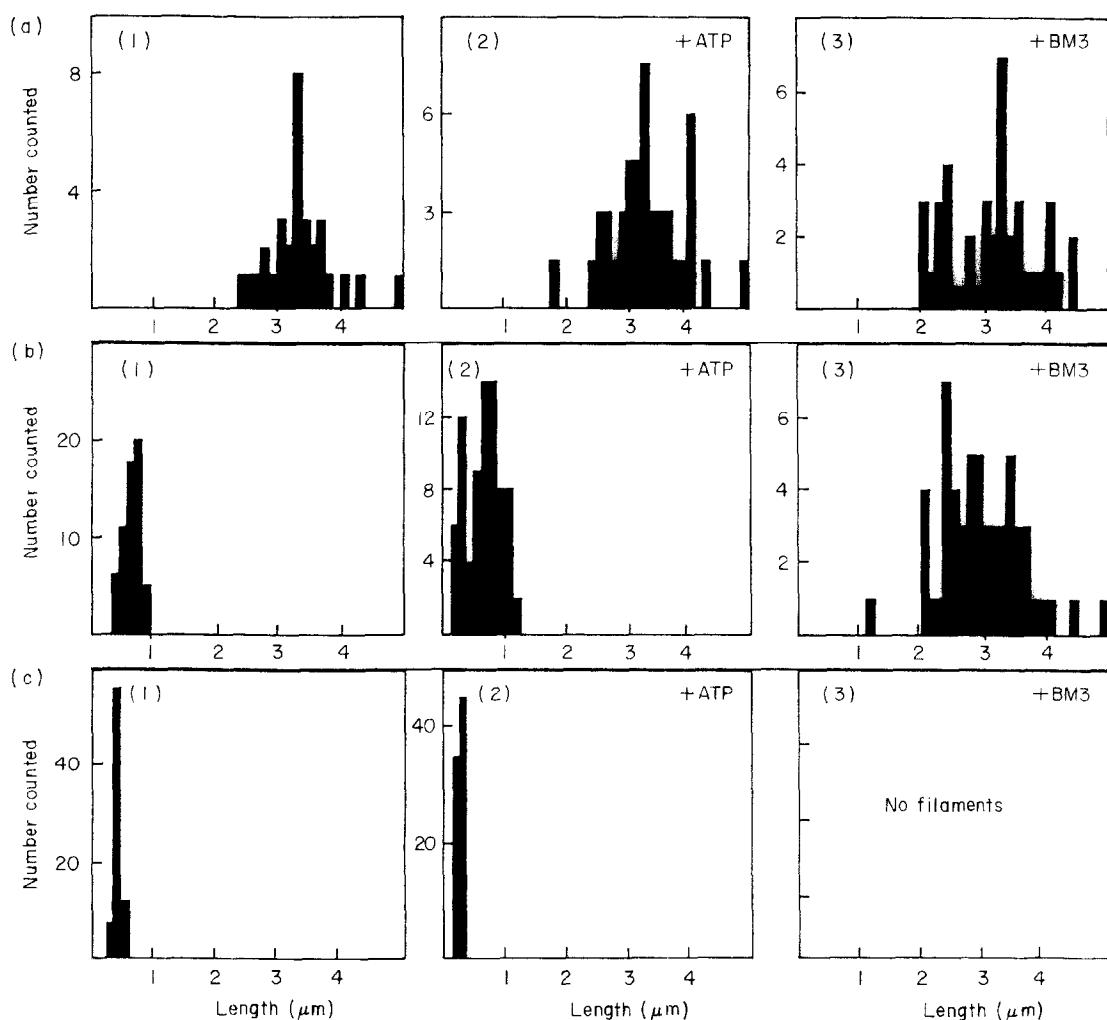
#### 4. Discussion

##### (a) Assembly of hybrid non-muscle/skeletal muscle myosin filaments

We observed that phosphorylated non-muscle myosin stabilizes copolymers with non-phosphorylated non-muscle myosin to disassembly by MgATP, but that skeletal muscle myosin has no effect.

The morphology of our skeletal/non-muscle myosin heteropolymers showed that myosins from different species and tissues can assemble together into a homogeneous set of filaments, in agreement with previous studies (Pollard, 1975; Kaminer *et al.*, 1976; Wachsberger & Pepe, 1980). In the absence of MgATP, the unimodal length distribution of the hybrid filaments suggests the presence of complementary assembly sites in these distinct myosins. However, at present we have no information on the distribution of either myosin within the filaments, for example whether the myosins are randomly distributed or whether the non-muscle myosin forms a bipolar region which may serve as a nucleating centre for skeletal myosin. The finding that the critical monomer concentration ( $C_c$ ) for the polymerization of non-muscle myosins are one to two orders of magnitude lower than those of skeletal myosins (Kendrick-Jones *et al.*, 1987) would support the latter suggestion. Further support is provided by the observation that small amounts of the non-muscle myosins have a dramatic effect on the size of these heteropolymers (Fig. 2). Thus, in the hybrid filaments the non-muscle myosin may form the antiparallel-packed nucleating zones whereas the skeletal myosin may be involved in parallel elongation from these nuclei. It has been demonstrated that in skeletal muscle myosin "native bare zone assemblages", i.e. short bipolar filaments, can serve as nucleators for filament assembly (Niederman & Peters, 1982).

Since non-phosphorylated non-muscle myosin filaments, unlike those of skeletal myosin, can be disassembled by MgATP under approximately physiological conditions *in vitro*, we investigated whether the putative interactions between skeletal myosin and non-muscle myosin molecules in the hybrid filaments would affect the disassembly of

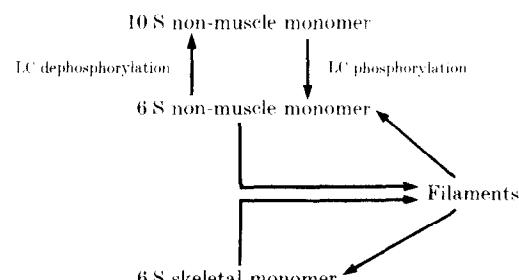


**Figure 8.** Effect of ATP and monoclonal antibody BM3 on the length of skeletal, brush border, and skeletal/brush border myosin filaments. (a) Skeletal myosin filaments, (b) skeletal/brush border myosin heteropolymers (molar ratio of skeletal/brush border myosin of 1:1), (c) brush border myosin filaments, in (1) the absence of MgATP; (2) the presence of MgATP, and (3) the presence of monoclonal antibody BM3. The myosins, or myosin/antibody mixtures (antibody/myosin molar ratio 3:1) were dialysed against 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.5 mM-EGTA, 0.2 mM-DTT, 20 mM-imidazole (pH 7.0), to form filaments. The lengths of the negatively stained filaments were measured as described in the legend to Fig. 1.

non-phosphorylated non-muscle myosin. In the presence of MgATP, we obtained a partial segregation of brush border myosin from the skeletal/brush border myosin hybrids, producing a heterogeneous set of filaments, with a bimodal length distribution (Fig. 8). It should be noted that under the conditions used only ~40% of the brush border conditions used only ~40% of the brush border myosin is disassembled by MgATP (Fig. 3); thus, the presence of short filaments and heteropolymers may be due to the fact that ~60% of brush border myosin remained in a filamentous state. Since skeletal myosin does not stabilize the non-muscle myosin (see Fig. 3) these results indicate that the non-muscle and skeletal monomers may disassemble and reassemble independently in the presence of MgATP, resulting in a mixture of hetero- and homopolymers. Only with the antibody BM3, which abolishes assembly of brush border myosin filaments, was there complete segregation of

brush border myosin from skeletal myosin. However, using thymus myosin, which is more soluble than brush border myosin (Fig. 3), we observed in the analytical ultracentrifuge an almost complete segregation of the two myosins in the presence of MgATP (Fig. 5).

These results may be explained by modifying our monomer-polymer equilibrium model for the polymerization of non-muscle myosins as follows:



The composition of the filaments (homo- or heteropolymers) depends on the concentrations of non-muscle and skeletal myosin monomers available for assembly. Light chain (LC) phosphorylation controls the monomer-polymer equilibrium in the non-muscle myosin by regulating the  $6\text{S} \rightleftharpoons 10\text{S}$  monomer transition (Kendrick-Jones *et al.*, 1987). Since the concentration of non-muscle myosin monomer is the same in the presence or absence of skeletal myosin (Fig. 3), skeletal myosin has no effect on the  $10\text{S} \rightleftharpoons 6\text{S} \rightleftharpoons$  polymer equilibria of non-muscle myosins.

(b) *Phosphorylated myosin stabilizes non-muscle myosin filaments*

Phosphorylated non-muscle myosin stabilizes co-operatively hybrid filaments composed mainly of non-phosphorylated myosin molecules. The molecular basis for this filament stabilization is unclear. The phosphorylated myosin molecules could act either by stabilizing the non-phosphorylated myosin molecules within the filaments and/or altering the  $10\text{S} \rightleftharpoons 6\text{S}$  equilibrium between folded and extended monomers. Since the stabilizing effect appears to be co-operative, we favour the idea that the phosphorylated myosin acts mainly at a filamentous level. Such a mechanism has been suggested in scallop striated muscle myosin, where it was proposed that changes in intermolecular contacts between myosin molecules, induced by  $\text{Ca}^{2+}$  binding to the light chains, co-operatively activate the whole thick filament (Vibert & Craig, 1985). Similarly, in *Acanthamoeba* myosin II it was suggested that regulation of the actin-activated ATPase by phosphorylation is exerted through intermolecular interactions within the filament (Kuznicki *et al.*, 1983).

If *in-vivo* light chain phosphorylation does control myosin filament assembly/disassembly, then the co-operative effects of phosphorylation we observed here *in vitro* may be a useful mechanism for maintaining stable myosin filaments and a continuous tension state in the cell, even at low levels of light chain phosphorylation.

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