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Actin and Myosin: Control of Filament Assembly [and Discussion]

J. A. Spudich, J. D. Pardee, P. A. Simpson, K. Yamamoto, E. R. Kuczmarski, L. Stryer and D. M. Shotton

Phil. Trans. R. Soc. Lond. B 1982 **299**, 247-261
doi: 10.1098/rstb.1982.0130

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Phil. Trans. R. Soc. Lond. B **299**, 247–261 (1982)
Printed in Great Britain

Actin and myosin: control of filament assembly

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Actin filaments, assembled from highly purified actin from either skeletal muscle or *Dictyostelium* amoebae, are very stable under physiological ionic conditions. A *small* and *limited* amount of exchange of actin filament subunits for unpolymerized actin or subunits in other filaments has been measured by three techniques: fluorescence energy transfer, incorporation of ^{35}S-labelled actin monomers into unlabelled actin filaments, and exchange of [^{14}C]ATP with filament-bound ADP. A 40 kDa protein purified from amoebae destabilizes these otherwise stable filaments in a Ca^{2+}-dependent manner. Myosin purified from *Dictyostelium* amoebae is phosphorylated both in the tail region of the heavy chain and in one of the light chains. Phosphorylation appears to regulate myosin thick-filament formation.

Introduction

After the discovery by Hatano & Oosawa (1966) that actin is a component of the cellular slime mould *Physarum polycephalum*, actin and myosin were demonstrated to exist in essentially all eukaryotic cells (for review see Pollard & Weihing 1974) and a wealth of information was obtained about the cellular localization of these proteins at different stages of growth and development (for review see Goldman *et al.* 1976). A primary characteristic of the contractile apparatus in non-muscle cells, unlike that in muscle, is the transient nature of the filamentous assemblies. These assemblies appear in the right place and at the right time for a particular form of movement to occur, and then the filaments disappear. The transient nature of these filamentous assemblies requires strict controls of their assembly and organization. A large number of cellular components involved in these controls are expected, and numerous accessory proteins that modulate actin or myosin assembly in a large number of cells have recently been discovered (for review see Schliwa 1981).

A primary emphasis of our laboratory has been biochemical and structural studies of actin and myosin from the cellular slime mould *Dictyostelium discoideum*. This eukaryotic cell can be easily cloned, grown in large amounts in a chemically defined medium, and manipulated genetically to examine mutants defective in motility. Furthermore, at one stage of the developmental cycle of the organism, the cells exhibit chemotaxis toward cyclic AMP. A detailed understanding of how this external signal is coupled to the intracellular contractile apparatus to cause the cell to move in a particular direction is one goal of work in our laboratory. We present here some of our recent results concerning the control of actin and myosin filament formation in *Dictyostelium*. Comparative experiments with skeletal muscle actin are also presented.

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Results and discussion

Dictyostelium actin

There appears to be only one major actin species in *Dictyostelium discoideum* (Uyemura *et al.* 1978; Vandekerckhove & Weber 1980), and it has many properties similar to actin purified from striated muscle (Woolley 1972; Spudich 1974; Spudich & Cooke 1975; Uyemura *et al.* 1978). Of particular interest is that under conditions of ionic strength and Mg^{2+} concentration that approximate intracellular conditions, the critical concentration C_a for polymerization of the purified *Dictyostelium* actin is only about 0.5 μM, whereas the total concentration of actin in the cell can be estimated to be about 200 μM (Spudich & Cooke 1975; Uyemura *et al.* 1978). Thus the inherent disposition of purified *Dictyostelium* actin, like muscle actin, is to be in the form of filaments. This observation raises two fundamentally important questions about actin filaments. First, how stable are actin filaments once they are formed? Are they rapidly turning over at steady state with a monomeric pool of actin, as would be suggested by a rapid 'treadmilling' process proposed in 1976 by Wegner, or are the monomers in the filament very slow to exchange with the monomeric pool of actin at steady state? Second, are there accessory proteins in *Dictyostelium* that can alter the assembly state of the actin?

Actin filaments are stable in conventional F buffer

A number of methods are available to examine the rate of exchange of monomers with filaments at steady state. A method of choice would involve marking the monomeric units within the filament such that their rate of exit from the filaments at steady state could be measured. An excellent assay that directly follows such exchange of the actin monomers uses fluorescence energy transfer (Stryer 1978). In the experiments reported here, we followed the quench in fluorescence of a donor probe as an acceptor-labelled actin monomer was brought into close proximity (figure 1). This methodology was first applied to actin filaments by Taylor

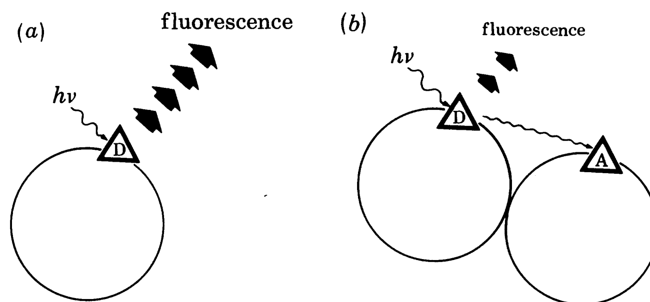

Figure 1. Fluorescence energy transfer as applied to studies of actin filament assembly. Actin monomers labelled with a donor fluorochrome exhibit a characteristic fluorescence emission when excited by light of the appropriate wavelength (*a*). The excitation energy can be transferred to acceptor-labelled monomers by resonance energy transfer if the donor-labelled and acceptor-labelled monomers are brought into close proximity (*b*). Such transfer results in reduction (quenching) in fluorescence intensity of the donor fluorochrome.

et al. (1981), who used fluorescein as a donor molecule, and rhodamine or eosin as an acceptor. We have continued to exploit this technology to answer some fundamental questions about the assembly and disassembly of actin filaments (Pardee *et al.* 1982). Most of the experiments in this report were carried out with highly purified muscle actin prepared as described by Spudich

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& Watt (1971), with additional purification as described by Pardee & Spudich (1982). Experiments that give essentially the same results (for example, see figure 5) have also been carried out with actin prepared from *Dictyostelium* by using the method described by Uyemura *et al.* (1978).

In these studies, *N*-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulphonate (IAENS) served as the donor fluorescent probe, and fluorescein isothiocyanate (FITC) as the acceptor. IAENS is known to label muscle actin specifically at Cys 373 (Tao & Cho 1979). This donor–acceptor

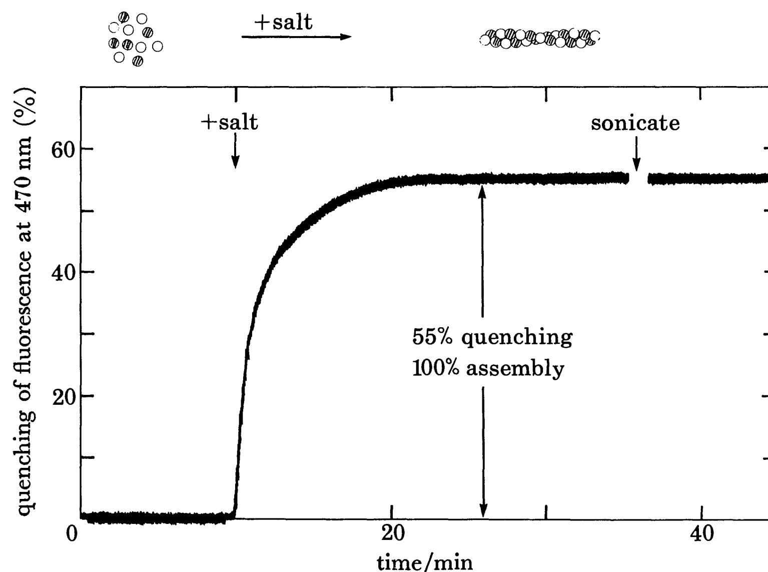

FIGURE 2. Actin assembly can be monitored by fluorescence energy transfer. Rabbit skeletal muscle IAENS-labelled G-actin at 0.2 mg ml^{-1} was mixed with 0.8 mg ml^{-1} FITC-labelled G-actin (both in 2 mM TES, pH 7.2, 1 mM ATP, 0.5 mM 2-mercaptoethanol, 50 μM $CaCl_2$ 50 μg ml^{-1} NaN_3) and fluorescence emission monitored at 470 nm. After a 10 min incubation at 25 °C to establish the zero quenching baseline, KCl and $MgCl_2$ were rapidly added to 0.1 M and 1.0 mM, respectively, and the increase in fluorescence quenching (decrease in fluorescence emission at 470 nm) was monitored. After completion of assembly, the sample was sonicated for 1 min on ice to ensure complete randomization of fluorescent label within filaments. No further increase in quenching was observed, indicating complete randomization by the co-assembly process; maximum quenching (55%) is obtained when all IAENS-labelled actin subunits are surrounded by FITC-labelled actin subunits within the filament structure.

pair has several attractive features. First, the fluorescence of the donor in the absence of acceptor is virtually unaltered by the conversion of G-actin to F-actin. Second, the emission maximum of the donor at 470 nm does not overlap the emission spectrum of the acceptor. Third, this donor–acceptor pair has an R_0 (50% transfer efficiency) distance of about 5 nm, a very favourable value. The fluorescence-labelled actins were characterized with regard to their kinetics of assembly, their critical concentrations at steady state, and their ability to hydrolyse ATP during assembly and at steady state. In all of these characteristics, the labelled actins behaved as the unlabelled actin.

The kinetics of assembly of a mixed population of monomers of donor-actin and acceptor-actin are shown in figure 2. At 10 min, salt was added to induce polymerization. The kinetics of assembly are rapid under these conditions and can be followed by the quenching of fluorescence that results from energy transfer between adjacent donor-labelled and acceptor-labelled actin units in the filament. It should be noted that separation of an adjacent donor–acceptor

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pair (*ca.* 5.5 nm apart) by a single unlabelled monomer would lead to almost no energy transfer because the donor–acceptor distance would then be 11 nm. This method is therefore exquisitely sensitive to the presence of donor and acceptor units in adjacent positions in the actin filament. Under the conditions of the experiment shown in figure 2, 100 % assembly (i.e., the maximum expected under these buffer conditions) corresponds to about 55 % fluorescence quenching. As

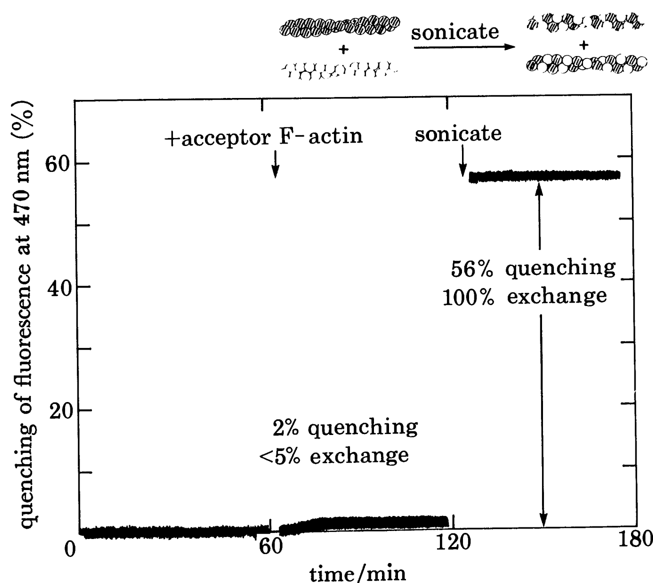

FIGURE 3. Actin monomer exchange between filaments is minimal without shear. Aliquots of IAENS-labelled, FITC-labelled and unlabelled rabbit muscle G-actin (all in 2 mM TES, pH 7.2, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 50 μM $CaCl_2$, 50 μg ml^{-1} NaN_3) were assembled separately by addition of 0.1 M KCl, 1 mM $MgCl_2$ and ATP to 1 mM. Baseline fluorescence quenching was established by mixing 0.2 mg ml^{-1} IAENS-labelled F-actin with 0.8 mg ml^{-1} unlabelled F-actin and monitoring fluorescence at 470 nm. After incubation for 60 min at 25 °C to establish a baseline spectrum, a separate portion of IAENS-labelled F-actin (0.2 mg ml^{-1}) was mixed with 0.8 mg ml^{-1} FITC-labelled F-actin, and the increase in fluorescence quenching (decrease in fluorescence emission at 470 nm) was monitored with time. The mixed filaments were then sonicated under conditions known to randomize completely the filament subunits. Sonication produced 56 % fluorescence quenching, the amount expected for 100 % exchange (see figure 2). The percentage subunit exchange was calculated from the fluorescence observed immediately after mixing donor-labelled and acceptor-labelled filaments ($F_{initial}$), at the end of the exchange period (F_{final}), and after sonication ($F_{sonicate}$) by using the relation

$$\text{subunit exchange} = \frac{F_{initial} - F_{final}}{F_{initial} - F_{sonicate}} \times 100\,\%.$$

Skeletal muscle actin was purified by the procedure of Spudich & Watt (1971), followed by DEAE chromatography (Pardee & Spudich 1982).

shown below, sonication of filaments causes a total randomization of the monomers. In figure 2 the filaments are completely randomized before sonication. Sonication therefore has no effect on the fluorescence intensity.

In figure 3 another type of fluorescence experiment is shown. In this case we wished to quantitate the level of subunit exchange between filaments. Here donor-labelled filaments are prepared separately from acceptor-labelled filaments and the two filament populations are mixed. Energy transfer should reflect the extent to which the monomeric pool is exchanging with the filaments, since exchange would result in randomization of the donor-labelled and acceptor-labelled monomers between the two filament populations. This is a very simple and

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direct measure of the rate of exchange of monomers with the filament subunits. The result of this experiment indicates that there is very little (less than 5 %) exchange of monomers with the filaments in an hour. Sonication of the mixed population of labelled filaments at 120 min resulted in the total randomization of the monomers (figure 3). Experiments conducted over a 6 h period revealed no increase in fluorescence quenching, indicating limited exchange (less than 5 %) between filaments at an actin concentration of 1 mg ml^{-1}. Thus purified actin filaments are very stable under these conditions (0.1 M KCl, 1 mM $MgCl_2$, 1 mM ATP, pH 7.2); there is very little exchange of monomers with the filament on a timescale of several hours.

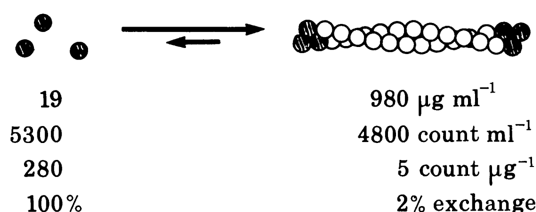

FIGURE 4. Exchange of [^{35}S]actin monomer into F-actin. A trace amount of [^{35}S]actin monomer was added to F-actin at 1.0 mg ml^{-1} in F-buffer (3 mM imidazole, pH 7.5 at 25 °C, 0.2 mM ATP, 0.2 mM dithiothreitol, 50 μg ml^{-1} NaN_3, 0.1 M KCl, 1 mM $MgCl_2$). After incubation for 3 h at 25 °C, F-actin was sedimented by centrifugation at 150000 *g* for 1.5 h at 25 °C. The distribution of radioactivity between actin filaments (pellet) and actin monomer (supernatant) was determined by liquid scintillation counting. The percentage exchange was calculated from the formula

$$\text{subunit exchange} = \frac{\text{specific radioactivity of F-actin}}{\text{specific radioactivity of G-actin}} \times 100\,\%.$$

In the example shown in the figure, even though considerable radioactivity was incorporated into F-actin, the amount of exchange is very small because of the large difference in the concentrations of actin monomer and filament populations.

These conclusions are consistent with experiments by Simpson & Spudich (1980) in which *Dictyostelium* actin was labelled biosynthetically with [^{35}S]methionine and the incorporation of labelled monomer by exchange into F-actin filaments was measured at steady state. Rapid incorporation of some of the labelled G-actin, probably into the ends of actin filaments, is observed (Simpson & Spudich 1980). However, because the monomer concentration is on the order of 10–20 μg ml^{-1} at steady state, only a small percentage of the total F-actin need exchange at the ends of the filaments to account for a large amount of radioactive G-actin incorporated (figure 4).

Yet another measure of subunit exchange, although indirect, is the incorporation of [^{14}C]ATP into unlabelled filaments (for review see Taylor 1972). All three approaches, fluorescence energy transfer, [^{35}S]G-actin incorporation, and [^{14}C]ATP incorporation, indicate that at all actin concentrations, a rapid ($t_{\frac{1}{2}} < 30$ min) *limited* exchange occurs, which reaches a plateau value that is plotted in figure 5. Indeed, the exchange as a function of the actin concentration is the same regardless of which of the three assays is used (figure 5). Furthermore, the same results are obtained with muscle actin and with *Dictyostelium* actin (figure 5). Thus three independent methods show that actin filaments are very stable under conventional F-buffer conditions, with restricted rapid exchange of a relatively small number of monomers.

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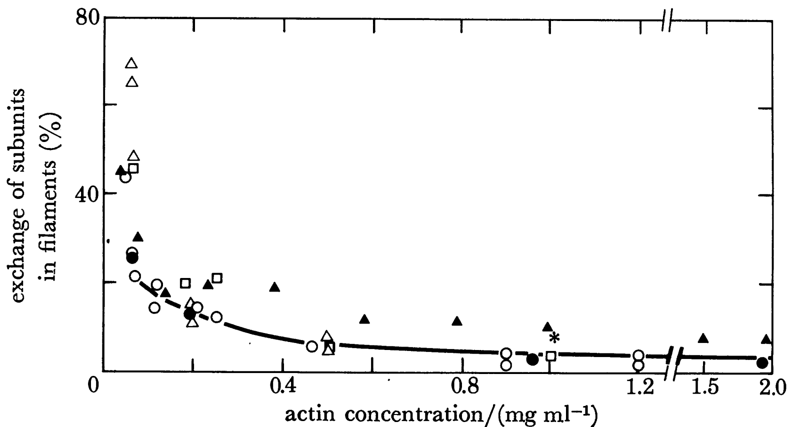

FIGURE 5. Extent of subunit exchange as a function of actin concentration. The extent of subunit exchange was determined by fluorescence energy transfer for muscle actin prepared in TES G-buffer containing 20 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (○); for muscle actin in 1 part buffer 'A' diluted into 4 parts PIPES buffer ($[K^+]$ = 8.4 mM, $[Mg^{2+}]$ = 2 mM, [ATP] = 0.44 mM final concentrations) (□); for *Dictyostelium* actin prepared in TES G-buffer containing 20 μM $MgCl_2$ in place of 50 μM $CaCl_2$, and assembled in 0.1 M KCl, 1 mM $MgCl_2$ (●). In these experiments exchange was measured by mixing donor-labelled (IAENS) filaments with acceptor-labelled (FM) filaments. Subunit exchange was also determined by the incorporation of ^{35}S-labelled *Dictyostelium* G-actin into unlabelled muscle F-actin in imidazole G-buffer containing 0.1 M KCl, 1 mM $MgCl_2$ and 1 mM ATP (△); and into *Dictyostelium* F-actin in imidazole G-buffer containing 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ATP (▲). Exchange of [^{14}C]ATP for F-actin-bound ADP is also shown (*).

Severin, a 40 kDa *protein from Dictyostelium, disassembles actin filaments in a* Ca^{2+}*-dependent manner*

Brown *et al.* (1982) purified to *ca.* 90 % homogeneity a 40 kDa protein from *Dictyostelium* which, in the presence of Ca^{2+}, disassembles actin filaments. Further purification has resulted in preparations that are 99 % pure, as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (figure 6) (Yamamoto *et al.* 1982). A number of approaches, including electron microscopy, viscometry, fluorescence anisotropy, DNase I inhibition assays, and sedimentation, suggest that this protein binds to actin filaments, severs them and remains bound, apparently to the 'barbed' end of the filaments (figure 7) (Yamamoto *et al.* 1982; A. Weeds & J. A. Spudich, unpublished). Considering the mechanism of action of this protein, we have named it severin. After fragmentation of filaments by severin, the amount of actin monomer increases to a new steady-state level and exchange of the monomer pool with filaments is significantly increased. These points are readily apparent in the experiments described below with the use of fluorescence energy transfer techniques (Yamamoto *et al.* 1982). All of the effects of severin have an absolute requirement for Ca^{2+}.

In the first experiment (figure 8), donor-labelled and acceptor-labelled monomers were mixed in G-buffer and a baseline fluorescence was established. As in the earlier experiment (figure 2), the addition of salt generates filaments that are totally randomized with respect to their donor–acceptor distribution. The amount of monomer at steady state is small (less than 40 μg ml−1) and the final extent of assembly (referred to as 100 % of that expected under these buffer conditions with the purified actin) results in 55 % quenching of donor fluorescence. At 30 min, severin was added in a 1:20 ratio to the actin. A limited, rapid loss in energy transfer occurred, which is explained by a decrease in the F:G ratio. Thus the amount of monomers at steady state increases upon addition of severin. Note that the increase in the monomer pool is

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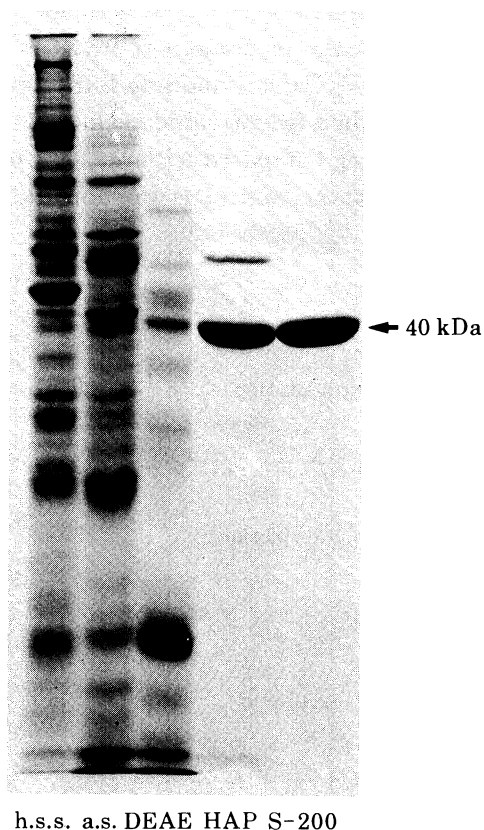

FIGURE 6. Severin, a 40 kDa protein from *Dictyostelium* that affects actin assembly, has been purified to 99% homogeneity. This sodium dodecyl sulphate (SDS) polyacrylamide gel shows fractions obtained during the purification of severin. *Dictyostelium* amoebae were lysed by sonication, and the proteins in a high-speed supernatant (h.s.s.) were fractionated by ammonium sulphate (60–80%) (a.s.). The suspended precipitate was dialysed against 2 mM triethanolamine buffer, pH 7.5, containing 0.2 mM DTT. DEAE cellulose (Whatman, DE-52), equilibrated with the same buffer, was mixed with the dialysate and unbound proteins were collected by filtration (DEAE). This filtrate was applied to an hydroxyapatite (BioRad) column, and bound proteins were eluted by a 0–0.3 M KCl gradient. Severin eluted from the column at a KCl concentration of 0.17 M (HAP). This HAP pool was concentrated by vacuum dialysis and applied to a Sephacryl S-200 (Pharmacia) column (S-200). The final product showed no apparent contaminants with a load of 8 μg on SDS–polyacrylamide gels.

probably not due to different critical concentrations at the two ends of an actin filament since under our experimental conditions the bulk of these actin filaments are not undergoing rapid vectorial exchange ('treadmilling'), and therefore the critical concentrations at the two ends would appear to be similar (Pardee *et al.* 1982). One possible explanation for this increase in the monomer pool is that the condensation mechanism for actin assembly proposed by Oosawa and his colleagues (Oosawa *et al.* 1959; Oosawa & Asakura 1975) is not precisely obeyed under these experimental conditions, and the amount of monomer at steady state does indeed increase as a function of the number of free ends. Another possibility is that binding of severin in some manner weakens the actin–actin associations along the filament, resulting in an increase in the critical concentration.

Note that in the presence of this amount of severin the new value for quenching at maximum assembly becomes about 40% for totally randomized filaments (figure 8); this value is pertinent for the next experiment.

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In figure 9, donor-labelled filaments were mixed with acceptor-labelled filaments at 60 min. Very little (less than 5%) exchange of monomers between the two filament populations is apparent over the next hour in the Ca^{2+}-containing buffer that activates severin. At 120 min, severin was added. This resulted in a *limited*, rapid exchange of monomers up to a plateau level of 57% exchange (23% quenching compared with *ca.* 40% quenching expected from figure 8 for 100% exchange). An important control is to sonicate the filaments to randomize the

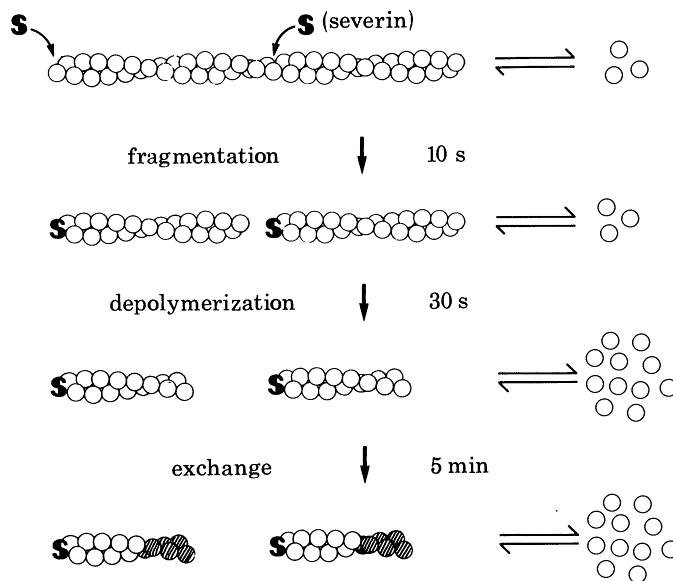

FIGURE 7. Proposed model for interaction of severin with actin filaments. By the use of low-shear viscometry and fluorescence energy transfer it has been possible to resolve kinetically several successive stages in the mechanism of interaction of severin with actin filaments (Yamamoto *et al.* 1982). Falling-ball viscometry indicates that actin filaments are fragmented within 30 s of the addition of severin in the presence of 0.2 mM Ca^{2+}. Electron microscopy of fragmented actin shows fragments of an approximately constant length, which is determined by the severin:actin ratio. Fluorescence anisotropy experiments with the use of IAENS-labelled severin demonstrate binding to actin fragments in the presence of Ca^{2+} but not in the presence of EGTA (R. G. Giffard & J. A. Spudich, unpublished observations). The protein has also been shown to block the addition of monomers to the 'barbed end' of actin filaments (A. Weeds & J. A. Spudich, unpublished observations). After fragmentation of filaments by severin, the monomer actin pool in equilibrium with filament fragments increases with a half-time of 20–30 s to a new equilibrium level (see figure 8). The rise in C_a observed by fluorescence energy transfer has also been quantitated by DNase I and sedimentation assays. The third stage of filament response to severin interaction is an increase in the amount of limited monomer–filament exchange. The increase in exchange follows the fragmentation and rise in C_a, and has a half-time of *ca.* 5 min.

monomers totally. In figure 9, sonication at *ca.* 180 min provides another 17% quenching, giving a total quenching of *ca.* 40%, in agreement with that expected for a totally randomized population in the presence of severin. We propose that a limited amount of exchange occurs at filament ends, as shown in the schematic diagram in figure 9. Only a small number of monomers at a filament end need be involved in this exchange. Ordinarily with filaments several micrometres long, this number represents less than 5% of the filament. When filaments are fragmented to numerous, very short filaments the same number of monomers exchanging per end represents a much larger proportion of the filament.

We conclude that severin, in the presence of Ca^{2+}, fragments otherwise stable filaments. This

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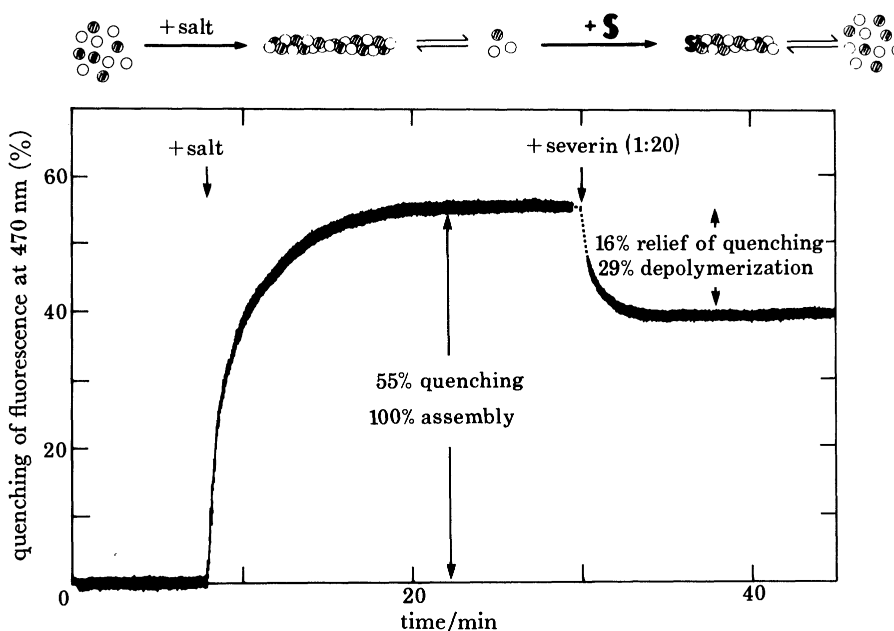

FIGURE 8. Actin filament fragmentation by severin induces an increase in the actin monomer pool size at steady state. A 10:1 ratio of rabbit skeletal muscle FITC–G-actin to IAENS–G-actin (both in 20 mM triethanolamine, 2 mM TES, pH 7.4, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol and 50 μg ml^{-1} NaN_3) was assembled by the addition of KCl (to 50 mM) and $MgCl_2$ (to 2 mM). The total actin concentration was 0.75 mg ml^{-1}. Assembly was followed by the increase in fluorescence quenching at 470 nm. Severin was added to the assembled actin (1:20) and the relief of fluorescence quenching monitored. Because of the large excess of acceptor-labelled actin in the co-assembled filaments (10 acceptors per donor), the production of actin filament fragments containing 10–20 subunits will not cause a decrease in observed quenching; significant reduction of fluorescence quenching occurs only upon disassembly of filaments to the size of monomers or dimers.

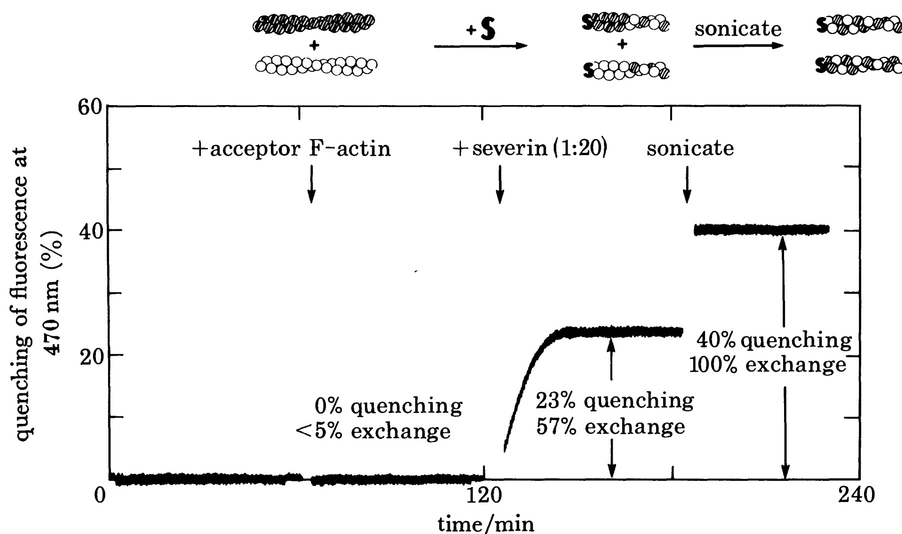

FIGURE 9. Severin enhances actin monomer exchange between filaments. Donor-labelled and acceptor-labelled rabbit muscle actin (in 20 mM triethanolamine, 2 mM TES, pH 7.4, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, and 50 μg ml^{-1} NaN_3) were assembled separately by the addition of KCl (to 50 mM) and $MgCl_2$ (to 2 mM). Donor–F-actin and acceptor–F-actin were mixed (0.1 mg ml^{-1} donor + 0.9 mg ml^{-1} acceptor) and fluorescence quenching was monitored at 470 nm. A 1:20 ratio of severin:actin was then added and the quenching monitored. After the plateau value for quenching was reached, the sample was sonicated to randomize completely the subunits between the donor and acceptor filaments (100 % exchange). Note that this type of fluorescence energy transfer experiment does not detect the rise in C_a after filament fragmentation; the result here indicates that after partial depolymerization of the filament fragments, 57 % of the remaining filament subunits exchange between the filament and monomer pools.

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produces a large number of short filaments, and therefore a large increase in the number of filament ends. We see a concomitant increase in the size of the monomer pool at steady state as well as an increase in the total amount of exchangeable monomers in the filament population.

Filament fragmentation increases the steady-state ATPase activity of F-actin

Filament subunit exchange is accompanied by hydrolysis of one ATP for each subunit exchange event (Martonosi *et al.* 1960; Oosawa & Kasai 1971). In the scheme shown in figure 10, most of the filament subunits are not available for exchange and the rate of ATP hydrolysis

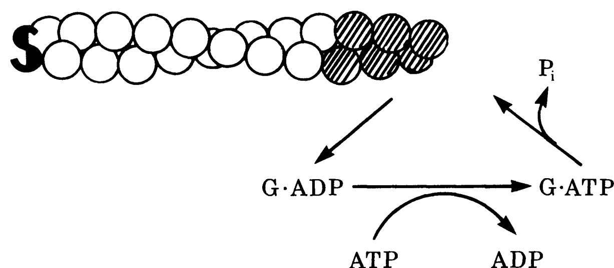

FIGURE 10. Model for steady-state ATP hydrolysis restricted to actin filament ends.

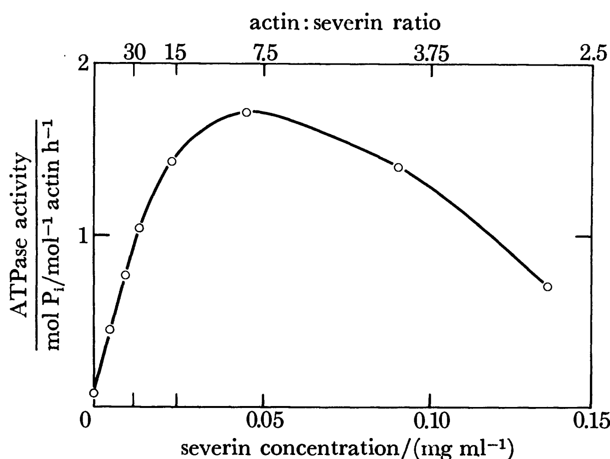

FIGURE 11. Effect of fragmentation on the ATPase activity of F-actin. The steady-state ATPase activity of rabbit muscle F-actin at 0.4 mg ml^{-1} was measured in F-buffer (20 mM triethanolamine, 2 mM TES, pH 7.4 at 25 °C, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 50 µg ml^{-1} NaN_3, 50 mM KCl, 2 mM $MgCl_2$) in the presence of increasing amounts of severin, by using the radiochemical assay described by Clarke & Spudich (1974). Severin alone exhibits no ATPase activity under these conditions.

is limited by the number of filament ends. Therefore, as the number of filament ends increases from fragmentation by severin, the rate of ATP hydrolysis should increase. The rate should be maximal at an actin:severin ratio where the number of subunits not available for exchange approaches zero. This ratio is expected to be on the order of 15:1 (Yamamoto *et al.* 1982). At lower actin:severin ratios the total amount of exchangeable actin decreases owing to an increasing proportion of actin subunits bound in tight non-exchangeable complexes with severin. The experimental results are consistent with this interpretation (figure 11). These results together with those presented above suggest strongly that the scheme presented in figure 7 for the mechanism of action of severin is correct.

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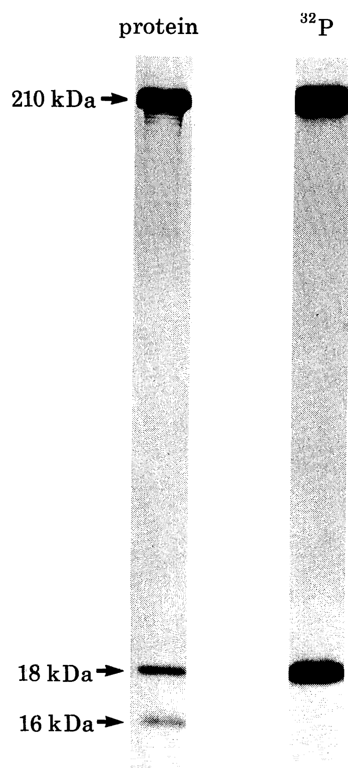

FIGURE 12. The heavy chain and one light chain of *Dictyostelium* myosin are phosphorylated *in vivo*. *Dictyostelium* amoebae were grown for four generations in [^{32}P]orthophosphate (15–20 μCi ml^{-1}) in a defined medium. Myosin was purified by modifications of the procedure of Mockrin & Spudich (1976) and the subunits separated on a 12% SDS–polyacrylamide gel (protein). The corresponding autoradiograph (^{32}P) demonstrates that the 210 kDa heavy chain and the 18 kDa light chain, but not the 16 kDa light chain, are phosphorylated *in vivo*.

Dictyostelium myosin

Myosin from *Dictyostelium* was first characterized by Clarke & Spudich (1974). In general, properties of *Dictyostelium* myosin resemble those of muscle myosin (Clarke & Spudich 1974; Mockrin & Spudich 1976). Kuczmarski & Spudich (1980) found that *Dictyostelium* myosin is phosphorylated *in vivo* on both the heavy chain and the 18 kDa light chain (figure 12); furthermore, *in vitro* transfer of the γ-phosphate from ATP to both the heavy chain and the 18 kDa light chain has been demonstrated by using partly purified *Dictyostelium* protein kinase preparations. It was of great interest to find that the heavy-chain phosphorylation site is in the tail portion of the myosin molecule (Peltz *et al.* 1981) because the tail is involved in the formation of thick filaments (Huxley 1963). In order to examine the effect of the heavy chain phosphorylation on myosin thick filament formation (Kuczmarski & Spudich 1980), it was first necessary to obtain a preparation of heavy-chain kinase that was sufficiently pure to be devoid of proteases that would otherwise modify the myosin by digestion. Similarly a phosphatase preparation that was free of proteases and that would remove the heavy-chain phosphate had to be obtained. We were able to obtain such preparations (Kuczmarski & Spudich 1982), which allowed us to prepare myosin with nearly 1 mol of phosphate per mole of heavy chain as well as myosin with no phosphate attached to the heavy chain (figure 13). These myosins were then compared for their ability to form filaments at various salt concentrations. A remarkable

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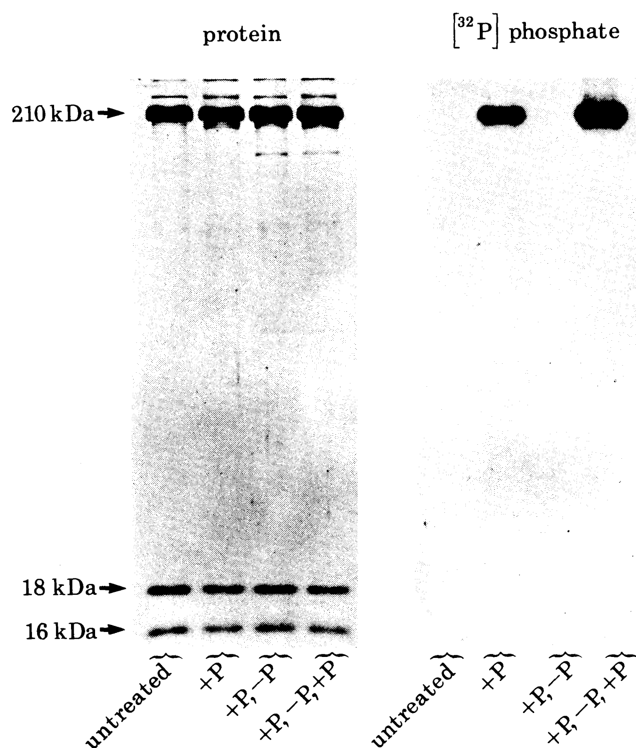

FIGURE 13. The heavy-chain phosphorylation site can be reversibly phosphorylated and dephosphorylated *in vitro*. An SDS–polyacrylamide gel (12%) is shown on the left and its autoradiograph on the right. Myosin was purified from amoebae grown in non-radioactive medium (untreated) and a portion of this myosin was phosphorylated *in vitro* (+P) using a partly purified heavy-chain kinase from *Dictyostelium* (Kuczmarski & Spudich 1982). Some of this phosphorylated myosin was treated with bacterial alkaline phosphatase to remove the phosphate (+P, −P) and a portion of the resulting myosin was then rephosphorylated (+P, −P, +P). The measured levels of *in vitro* phosphorylation (moles of [32P]phosphate added per mole of heavy chain) were *ca.* 0.3 for the first phosphorylation (or *ca.* 0.6 assuming an endogenous phosphorylation of *ca.* 0.3), 0.06 for the dephosphorylated myosin and 0.6 for the rephosphorylated myosin. Note that there was no proteolysis of myosin subunits and that only the heavy chains were phosphorylated.

difference in the ability to form thick filaments was observed at 50–100 mM KCl, a range estimated to be near physiological in amoebae of *Dictyostelium*. Phosphorylation of the myosin heavy chain inhibited the ability of the myosin to form thick filaments (figure 14). At about 0.1 M KCl, complete phosphorylation (about 1 mol phosphate per mole of heavy chain) essentially eliminated thick-filament formation, whereas partial phosphorylation (about 0.4 mol phosphate per mole of heavy chain) simply reduced the amount of thick filament formation. Nearly all of the myosin formed thick filaments when the phosphate was removed (less than 0.1 mol phosphate per mole of heavy chain).

The extent to which this control mechanism operates in other cells remains to be determined. It is important to note that we would not have known that the heavy chain is phosphorylated in *Dictyostelium* without having grown the cells in [32P]orthophosphate and purified the myosin from such labelled cells. It seems likely that many organisms have the capability of phosphorylating and dephosphorylating the myosin tail, but further studies are necessary to reveal the extent to which this is a general phenomenon.

Interestingly, Rahmsdorf *et al.* (1978) found that extracts made from *Dictyostelium* amoebae that had been exposed to external pulses of cyclic AMP, the chemotactic agent for these cells,

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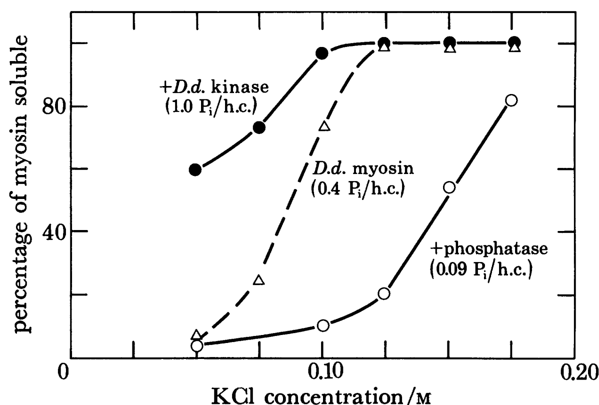

FIGURE 14. Heavy-chain phosphorylation inhibits myosin filament assembly. Myosin at 0.2 mg ml^{-1} was incubated in 10 mM Tris–HCl, pH 7.5, 0.1 mM dithiothreitol and 0.1 mM EDTA at various KCl concentrations. Myosin that assembled into thick filaments was removed by centrifugation (120000 *g* for 6 min in a Beckman Airfuge), and the remaining soluble myosin was measured by the method of Bradford (1976). The extent of phosphorylation (moles of phosphate per mole of heavy chain (h.c.)) for untreated, phosphorylated and dephosphorylated myosins are indicated in the figure.

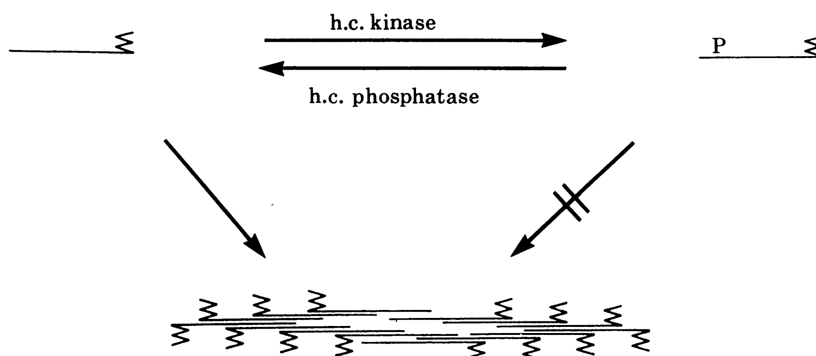

FIGURE 15. Control of myosin assembly by phosphorylation. The extent of myosin heavy chain phosphorylation is regulated by specific heavy chain (h.c.) kinase and phosphatase. Dephosphorylation of the myosin promotes assembly into thick filaments at physiological ionic strengths.

show an enhanced activity for phosphorylating a protein that co-migrates with the myosin heavy chain on SDS–polyacrylamide gels. Thus phosphorylation of myosin may prove to be an intermediate step between the external cyclic AMP signal and the extension of pseudopodia that results in directed cell movement.

SUMMARY AND CONCLUDING REMARKS

We have shown by three independent methods that actin filaments are very stable under conventional F-buffer conditions, with restricted rapid exchange of a relatively small number of actin monomers. Stoichiometric binding of an accessory protein of 40 kDa, isolated from *Dictyostelium* amoebae, alters the state of actin assembly. This protein, severin, fragments actin filaments in a Ca^{2+}-dependent manner. Current studies reveal a mechanism of control of assembly of *Dictyostelium* myosin filaments that is very different from that observed for actin filaments. From our experimental results it is likely that reversible phosphorylation of the tail region of the myosin heavy chain controls the assembly state. Further work may elucidate other

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proteins, both enzymes and stoichiometrically associating factors, which further modify the assembly state of both actin and myosin. Other investigators have purified actin gelation factors from *Dictyostelium* (Hellewell & Taylor 1979; Condeelis 1981), and a protein that causes actin filaments to form bundles has been reported (Spudich & Cooke 1975; Cooke *et al.* 1976).

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Discussion

D. M. Shotton (*Department of Zoology, University of Oxford, U.K.*). This involvement of myosin thick filaments in local pseudopod extension implies a frontal *contraction* mechanism. How does this square with Lance Taylor's results, which suggest that pseudopod extension is driven by hydrostatic pressure generated by tail-end contraction, and is made possible by a locally frontal *solation* of the actin microfilament cortical gel, without the involvement of myosin?

J. A. Spudich. There is too little factual information to answer Dr Shotton's question satisfactorily. I spoke of the possibility that the cAMP signal causes filament assembly, which could lead to contraction and thus to pseudopod formation (a frontal contraction model). However, one could equally argue that local relaxation of a contracted cortical shell of filaments, perhaps by filament disassembly, could give rise to a pseudopod, the relaxation allowing the cell to bulge out in that region to relieve a positive hydrostatic pressure within the cell (a tail-end contraction model). In some ways, I find this model more attractive than the former, but a great deal more information is required to distinguish between them. Another consideration is that the evidence from Lance Taylor and his colleagues for a tail-end contraction model stems primarily from studies on the giant amoebae. *Dictyostelium* amoebae more closely resemble mammalian cells in culture in their patterns of movement and shape changes. It may prove true that these cells move by way of frontal contraction whereas others such as the giant amoebae move by tail-end contraction.

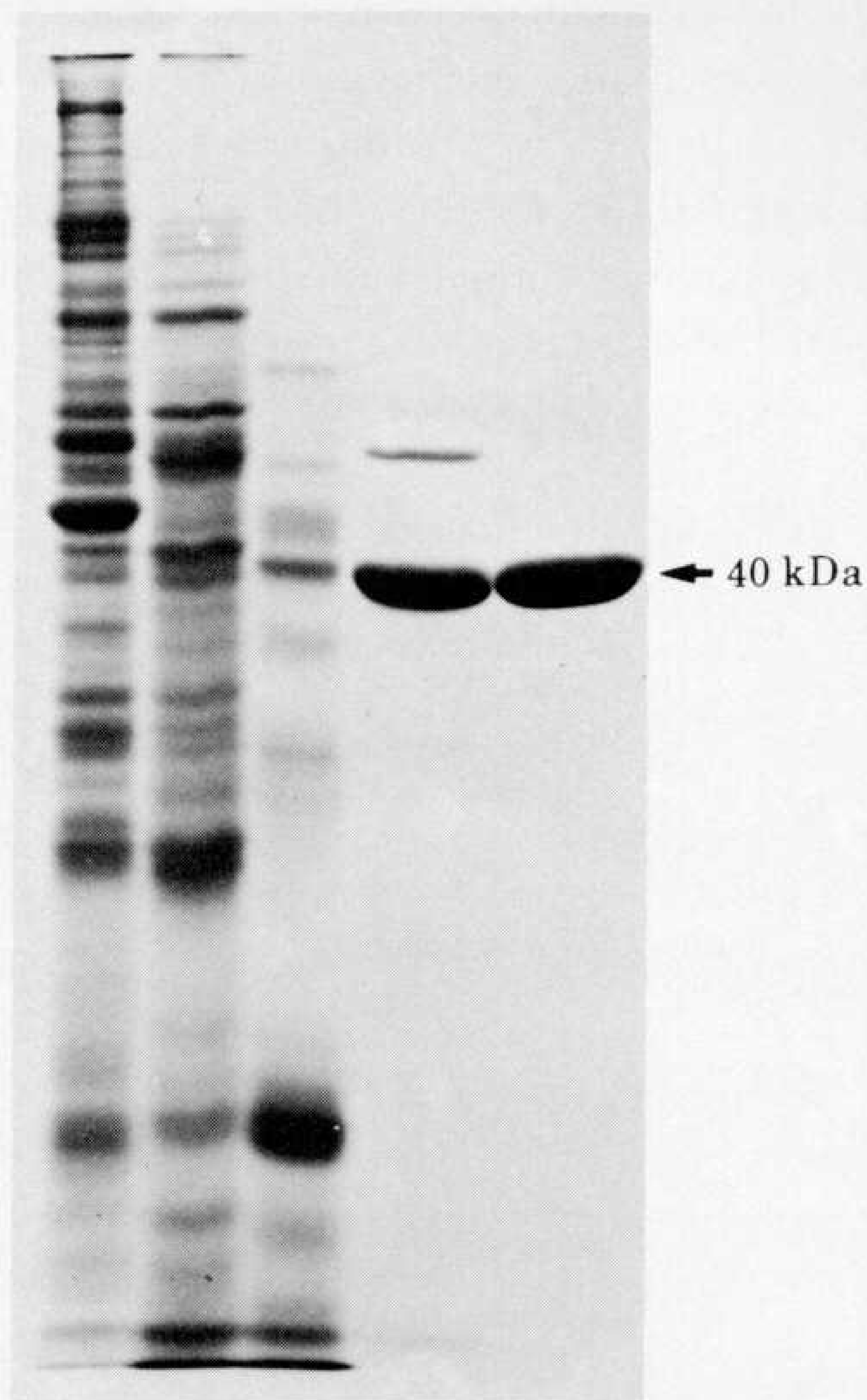

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FIGURE 6. Severin, a 40 kDa protein from *Dictyostelium* that affects actin assembly, has been purified to 99% homogeneity. This sodium dodecyl sulphate (SDS) polyacrylamide gel shows fractions obtained during the purification of severin. *Dictyostelium* amoebae were lysed by sonication, and the proteins in a high-speed supernatant (h.s.s.) were fractionated by ammonium sulphate (60–80%) (a.s.). The suspended precipitate was dialysed against 2 mM triethanolamine buffer, pH 7.5, containing 0.2 mM DTT. DEAE cellulose (Whatman, DE-52), equilibrated with the same buffer, was mixed with the dialysate and unbound proteins were collected by filtration (DEAE). This filtrate was applied to an hydroxyapatite (BioRad) column, and bound proteins were eluted by a 0–0.3 M KCl gradient. Severin eluted from the column at a KCl concentration of 0.17 M (HAP). This HAP pool was concentrated by vacuum dialysis and applied to a Sephacryl S-200 (Pharmacia) column (S-200). The final product showed no apparent contaminants with a load of 8 μg on SDS–polyacrylamide gels.

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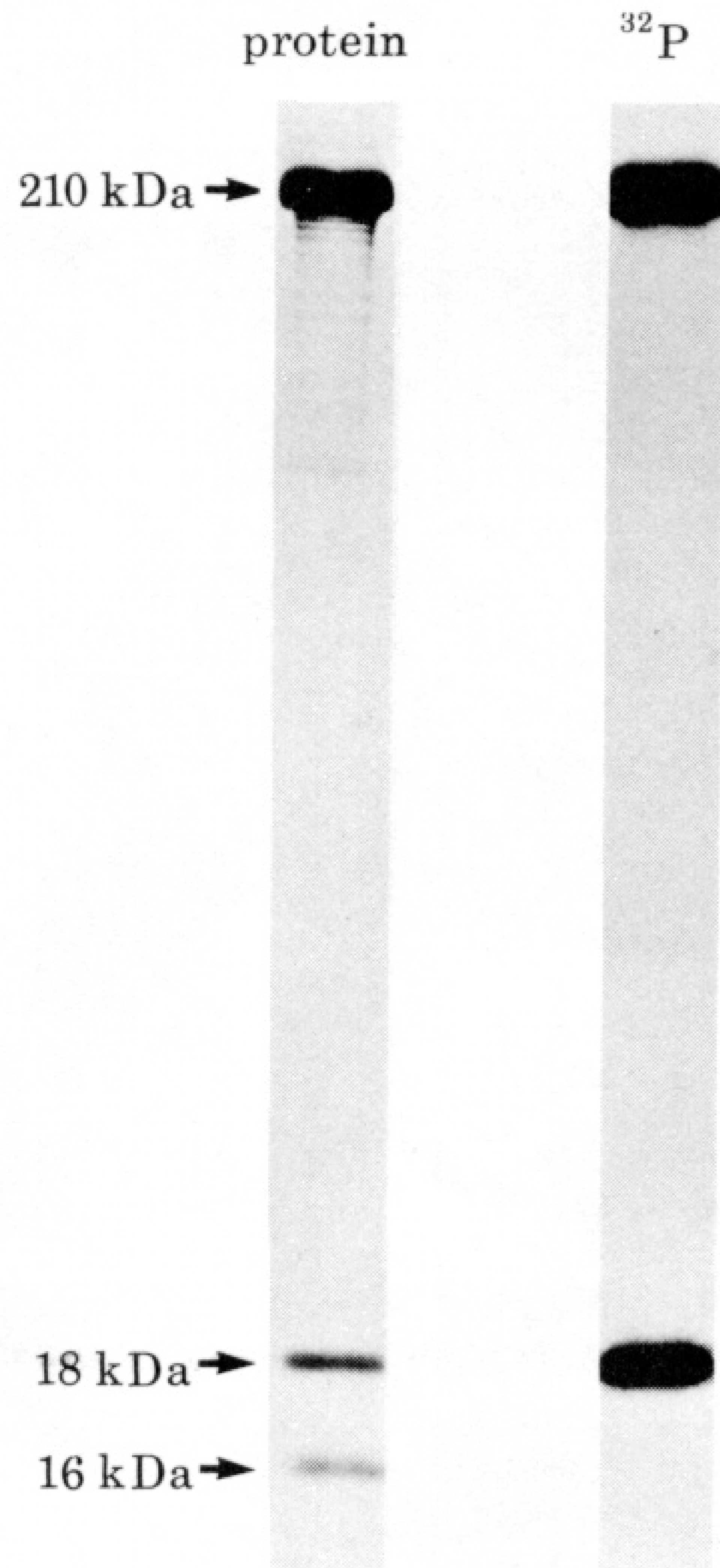

FIGURE 12. The heavy chain and one light chain of *Dictyostelium* myosin are phosphorylated *in vivo*. *Dictyostelium* amoebae were grown for four generations in [^{32}P]orthophosphate (15–20 μCi ml^{-1}) in a defined medium. Myosin was purified by modifications of the procedure of Mockrin & Spudich (1976) and the subunits separated on a 12% SDS–polyacrylamide gel (protein). The corresponding autoradiograph (^{32}P) demonstrates that the 210 kDa heavy chain and the 18 kDa light chain, but not the 16 kDa light chain, are phosphorylated *in vivo*.

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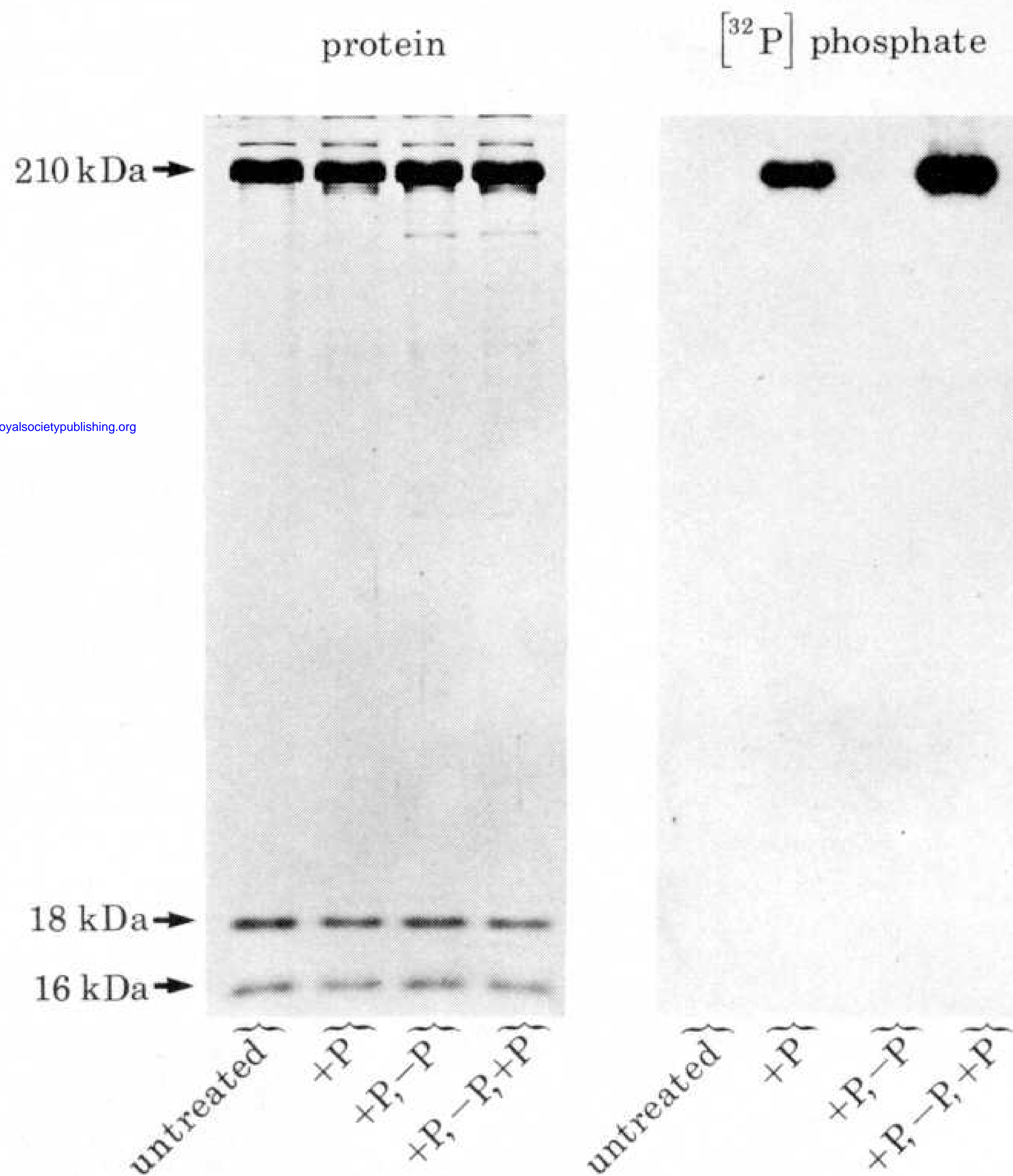

FIGURE 13. The heavy-chain phosphorylation site can be reversibly phosphorylated and dephosphorylated *in vitro*. An SDS–polyacrylamide gel (12 %) is shown on the left and its autoradiograph on the right. Myosin was purified from amoebae grown in non-radioactive medium (untreated) and a portion of this myosin was phosphorylated *in vitro* (+P) using a partly purified heavy-chain kinase from *Dictyostelium* (Kuczmarski & Spudich 1982). Some of this phosphorylated myosin was treated with bacterial alkaline phosphatase to remove the phosphate (+P, −P) and a portion of the resulting myosin was then rephosphorylated (+P, −P, +P). The measured levels of *in vitro* phosphorylation (moles of [^{32}P]phosphate added per mole of heavy chain) were *ca.* 0.3 for the first phosphorylation (or *ca.* 0.6 assuming an endogenous phosphorylation of *ca.* 0.3), 0.06 for the dephosphorylated myosin and 0.6 for the rephosphorylated myosin. Note that there was no proteolysis of myosin subunits and that only the heavy chains were phosphorylated.

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