

Control of Actin Filament Length by Phosphorylation of Fragmin-Actin Complex

Kiyoshi Furuhashi and Sadashi Hatano

Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Abstract. Fragmin is a Ca^{2+} -sensitive F-actin-severing protein purified from a slime mold, *Physarum polycephalum* (Hasegawa, T., S. Takahashi, H. Hayashi, and S. Hatano. 1980. *Biochemistry*. 19:2677-2683). It binds to G-actin to form a 1:1 fragmin/actin complex in the presence of micromolar free Ca^{2+} . The complex nucleates actin polymerization and caps the barbed end of the short F-actin (Sugino, H., and S. Hatano. 1982. *Cell Motil.* 2:457-470). Subsequent removal of Ca^{2+} , however, hardly dissociates the complex. This complex nucleates actin polymerization and caps the F-actin regardless of Ca^{2+} concentration. Here we report that this activity of fragmin-actin complex can be abolished by phosphorylation of actin of the complex. When crude extract from *Physarum* plasmodium was incubated with 5 mM ATP and 1 mM EGTA, the ac-

tivities of the complex decreased to a great extent. The inactivation of the complex in the crude extract was not observed in the presence of Ca^{2+} . In addition, the activities of the complex inactivated in the crude extract were restored under conditions suitable for phosphatase reactions. We purified factors that inactivated fragmin-actin complex from the crude extract. These factors phosphorylated actin of the complex, and the activities of the complex decreased with an increased level of phosphorylation of the complex. These factors, termed actin kinase, also inactivated the complex that capped the barbed end of short F-actin, leading to elongation of the short F-actin to long F-actin. Thus the length of F-actin can be controlled by phosphorylation of fragmin-actin complex by actin kinase.

It is considered that actin-binding or actin regulatory proteins play key roles in dynamic behaviors of actin cytoskeletons in nonmuscle cells (9, 14, 22, 29). Actin organizes into bundles of F-actin in plasmodium of a slime mold, *Physarum polycephalum*. These bundles could be observed as birefringent fibers under a sensitive polarizing microscope. They appear and disappear in accordance with the contraction-relaxation cycle of plasmodium (12). Fragmin (6, 8, 27), profilin (21), and a high molecular weight actin-binding protein (25) have been purified from the plasmodium and characterized in detail. Like other F-actin-severing proteins (gelsolin [28], severin [3], villin [2], etc.; see also reference 22), fragmin severs F-actin to produce shorter filaments in the presence of Ca^{2+} of more than 10^{-6} M (6, 8, 24). It binds to G-actin to form fragmin-actin complex. The fragmin-actin complex becomes a nucleus for actin polymerization and caps the barbed (or fast-growing) end of the short F-actin, so that annealing of the short F-actin is inhibited (24). Because these activities of fragmin are regulated by the physiological concentration of Ca^{2+} , fragmin may be responsible for regulating the actin organization. However, reversal of these fragmin activities is not achieved by only removal of Ca^{2+} . Fragmin bound to actin in the presence of Ca^{2+} hardly dissociated even when Ca^{2+} was removed so as to be $<10^{-6}$ M. A prolonged incubation (on the order of days) of fragmin-actin complex with EGTA is required for dissociation of the complex.

It has been recently reported that actin or actin regulatory

proteins could be regulated by polyphosphoinositides or phosphorylation. Profilin-actin and gelsolin-actin complexes were found to be dissociated into their components by phosphatidylinositol 4,5-bisphosphate (PIP_2) (13, 16). Sonobe et al. (23) showed that *Amoeba* G-actin could be phosphorylated by *Amoeba* actin kinase, and the phosphorylated G-actin was incapable of polymerization. Maruta et al. (19) isolated an actin regulatory protein from *Physarum plasmodium*, which was the complex of capping proteins termed Cap 42 (a) and Cap 42 (b). They reported that the capping activity of the complex was regulated by phosphorylation of Cap 42 (b) by an endogenous kinase (17).

In this paper we examined effects of phosphorylation on the activities of fragmin-actin complex. Our results demonstrate that fragmin-actin complex can be phosphorylated by endogenous protein kinases, and their nucleation and capping activities are abolished by the phosphorylation of the complex. These results suggest that the organization of the actin cytoskeleton in the plasmodium may be controlled by phosphorylation of the complex.

Materials and Methods

Culture of Plasmodia

Plasmodium of a myxomycete, *Physarum polycephalum*, was cultured by the method of Camp (4) using the modifications by Hatano and Tazawa (11).

Buffers

The following buffers were used. TE buffer: 0.1 mM EGTA and 10 mM Tris-HCl, pH 8.2. TEL β buffer: 0.1 mM EGTA, 10 mM β -mercaptoethanol, 1 μ g/ml leupeptin, and 10 mM Tris-HCl, pH 8.2. Pi buffer: 2 mM DTT, 10 mM β -glycerophosphate, 5 mM NaF, 2 mM pyrophosphate, 0.2 mg/ml leupeptin, 4 mM MgCl₂, 0.1 mM PMSF, and 30 mM Tris-HCl, pH 8.2. dePi buffer: 0.1 mM EGTA, 2 mM DTT, 2 mM MgCl₂, 0.2 mg/ml leupeptin, 0.2 mM PMSF and 30 mM Tris-HCl, pH 8.2.

Crude Extract

Fresh plasmodia (10–200 g) were homogenized in 2 vol of an extraction solution containing 15% sucrose, 45 mM EGTA, 0.15 mM PMSF, 1.5 mM DTT, 0.1 mg/ml leupeptin, and 30 mM Tris-HCl, pH 8.2, and extracted for 30 min at 4°C. The homogenate was centrifuged at 80,000 g for 90 min and extracted proteins were precipitated by ammonium sulfate of 80% saturation. The precipitate was dialyzed against TEL β buffer and centrifuged at 15,000 g. The supernatant was used as crude extract.

Proteins

Physarum G-actin was purified as previously described (10). Muscle G-actin was extracted from acetone-dried rabbit skeletal muscle prepared by the method of Ebashi and Ebashi (5).

Free fragmin in the crude extract was fractionated by the DEAE-cellulose column chromatography as described previously (6). Free fragmin was eluted at KCl concentration from 40 to 50 mM. The fractions containing fragmin were dialyzed against TE buffer, and applied to a DEAE-TOYOPEARL 650 column (1.6 \times 10 cm) equilibrated with the same buffer using fast performance liquid chromatography (FPLC¹; LKB Instruments, Inc., Gaithersburg, MD). The retained proteins were eluted with a linear gradient of 0–70 mM KCl at the flow rate of 1 ml/min. Free fragmin was eluted at 35 mM KCl. The fragmin fraction was applied to a Sephacryl S-300 HR column (1.6 \times 50 cm) equilibrated with TE buffer containing 50 mM KCl, and eluted at the flow rate of 1 ml/min (FPLC). Fragmin was eluted at the elution volume between 64 and 74 ml. The fragmin fraction was dialyzed against 0.6 M KCl and 20 mM K-phosphate, pH 6.8, applied to a hydroxylapatite column (0.8 \times 5 cm) equilibrated with the same buffer, and eluted with 0.6 M KCl and 50 mM K-phosphate, pH 6.8, at the flow rate of \sim 20 ml/h. 12 mg of free fragmin were purified from 350 g of the plasmodium.

Two kinds of fragmin-actin complexes were used in this study. "Purified complex" was prepared as follows. The fragmin-actin complex in crude extract was fractionated by DEAE-cellulose column chromatography (6). The complex eluted at 150–200 mM KCl was purified by gel filtration using AcA 44 followed by hydroxylapatite column chromatography according to the method described previously (8). The complex fractions were dialyzed against 50 mM KCl and 10 mM Tris-HCl, pH 8.2, and applied to a Sephacryl S-300 HR column (1.6 \times 50 cm) at the flow rate of 1 ml/min (FPLC). The complex was eluted at the elution volume between 59 and 66 ml. These fractions were directly applied to a DEAE-TOYOPEARL 650 column (1.6 \times 10 cm) equilibrated with 100 mM KCl and 10 mM Tris-HCl, pH 8.2, and eluted with a linear gradient of 100–200 mM KCl (FPLC). The complex was eluted at \sim 130 mM KCl. The flow rate was 1 ml/min. 9.4 mg of the complex were obtained from 350 g of the plasmodium. "Reconstituted complex" was prepared by incubating purified fragmin (12 mg) and *Physarum* G-actin (35 mg) in a solution containing 0.5 mM CaCl₂, 0.1 mM ATP, 0.2 mM DTT, and 30 mM Tris-HCl, pH 8.2. After incubation for 10 min at 0°C, the proteins were applied to a DEAE-TOYOPEARL 650 column (1.6 \times 10 cm) equilibrated with 100 mM KCl and 10 mM Tris-HCl, pH 8.2, and eluted with a linear gradient of 100–200 mM KCl (FPLC). The complex was eluted at \sim 130 mM KCl. The flow rate was 1 ml/min. The reconstituted complex was completely separated from unbound G-actin. 18 mg of the complex were recovered as the reconstituted complex.

DEAE-Cellulose Column Chromatography of Capping Activity in Crude Extract

The crude extracts were applied to DEAE-cellulose columns equilibrated with TE buffer. Retained proteins were eluted with a linear gradient of 0–250 mM KCl in TE buffer. Each fraction was incubated with muscle G-actin (\sim 1 mg/ml) in a solution containing 0.2 mM CaCl₂ and 10 mM Tris-

HCl, pH 8.2 for 1 min at 4°C, and then G-actin was polymerized on addition of MgCl₂ (2 mM) at 25°C. After 15 min the flow birefringence of F-actin was measured. As mentioned above, fragmin-actin complex becomes a nucleus for actin assembly and caps the barbed end of F-actin to form short F-actin. Therefore, the flow birefringence of the short F-actin becomes lower than that of F-actin polymerized in the absence of the complex. We defined this decrease in the flow birefringence of F-actin as "capping activity of fragmin-actin complex."

Purification of Factors That Inactivate Fragmin-Actin Complex

Crude extract from fresh plasmodia (50 g) was applied to a DEAE-cellulose column (1.5 \times 25 cm) equilibrated with TEL β buffer. Samples (20–40 μ l) of each fraction were assayed for activity in the inactivation of fragmin-actin complex. Assays were conducted in 60 μ l of 0.2 mg/ml purified complex, 5 mM ATP, 1 mM EGTA, and Pi buffer. The sample solutions were incubated for 10 min at 25°C, and then 60 μ l of 2 mg/ml muscle G-actin was added to each solution. After incubation for 15 min at 25°C, the flow birefringence of the solutions was determined. F-actin polymerized in the absence of fragmin-actin complex is longer than that in its presence. Inactivation of the complex results in an increase in the flow birefringence of the F-actin solution. We defined this increase in the flow birefringence as the "activity of factors that inactivate fragmin-actin complex." The factors were eluted at \sim 100 mM KCl from the DEAE-cellulose column, and then were purified by column chromatographies using FPLC. After the fractions containing the factors were diluted to the KCl concentration of 50 mM, they were applied to a DEAE-TOYOPEARL 650 column (1.5 \times 10 cm) equilibrated with TEL β buffer, eluted with a linear gradient of 50–150 mM KCl. The factors were eluted at \sim 100 mM KCl at the flow rate of 1 ml/min. These fractions were applied to a S-300 HR column (1.6 \times 50 cm) equilibrated with a TEL β buffer solution containing 150 mM KCl at the flow rate of 0.5 ml/min. The factors were eluted at the elution volume between 60 and 66 ml, which corresponded to that of the molecular mass of 110 kD. The fractions containing the factors were diluted to the KCl concentration of 50 mM, applied to a Mono Q column (0.5 \times 5 cm) equilibrated with TEL β buffer containing 50 mM KCl, and eluted with a linear gradient of 50–150 mM KCl at the flow rate of 0.5 ml/min. The factors were eluted at \sim 100 mM KCl (Fig. 2). The concentration of the finally purified factors was too low to determine the protein concentration.

Phosphorylation of Fragmin-Actin Complex by Purified Factors That Inactivate Complex

Purified fragmin-actin complex (0.64 mg/ml), reconstituted fragmin-actin complex (0.64 mg/ml) and G-actin (0.32 mg/ml) were incubated with the factors in Pi buffer (240 μ l) containing 0.4 mM ATP (25 μ Ci [γ -³²P]ATP) and 1 mM EGTA for 1 h at 25°C. Then the solutions were diluted to 1 ml with TE buffer. 6 M urea and 1 mM EGTA were added to each solution. After incubation for 1 h at 0°C, the samples were applied to DE-52 columns (1 ml) equilibrated with TE buffer. After washing with TE buffer, fragmin and actin were recovered by stepwise elution with KCl concentration from 0 to 70 mM and from 120 to 300 mM, respectively. The eluted proteins were subjected to SDS-PAGE and autoradiography (23).

We quantified ³²P incorporated into actin of the complex in the following way. Purified complex (0.2 mg/ml) and reconstituted complex (0.2 mg/ml) were phosphorylated by the factors in Pi buffer containing 1 mM EGTA and 0.2 mM ATP (25 μ Ci [γ -³²P]ATP) at 25°C. After phosphorylation reaction each sample was subjected to SDS-PAGE. The gel was stained by Coomassie brilliant blue. A part of the complex was cut out from the gel, and then the radioactivity of the complex was counted with a liquid scintillation counter (LS 9000; Beckman Instruments, Inc., Palo Alto, CA).

The capping activity of the phosphorylated complex was also measured as follows. After phosphorylation reaction, 40 μ l of 2.5 mg/ml muscle G-actin was added to 60 μ l of the sample solution containing 0.2 mg/ml the phosphorylated complex. After incubation for 6 min, the flow birefringence was measured.

Electron Microscopy

Samples were mounted on grids covered with carbon-coated collodion film and negatively stained with 1% uranyl acetate. Electron micrographs were taken at a direct magnification of 15,000 with a JEM 100-C electron microscope operated at 80 KV.

1. Abbreviation used in this paper: FPLC, fast performance liquid chromatography.

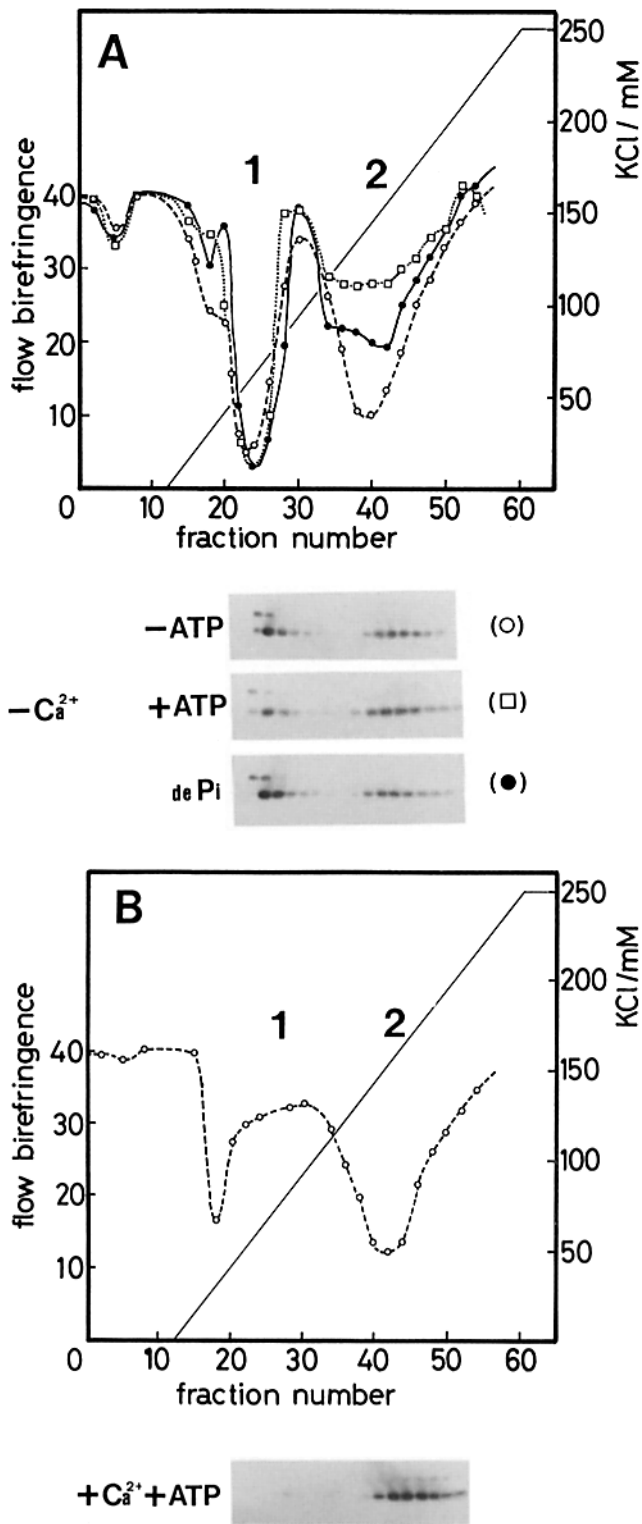


Figure 1. DEAE-cellulose column chromatography of capping activity in crude extract. Extract from fresh plasmodia (10 g) was divided into four parts. (A) Extract 1 and 2 were incubated with Pi buffer solutions containing 1 mM EGTA for 10 min at 25°C in the absence (open circles, -ATP) and the presence (open squares, +ATP) of 5 mM ATP. Extract 3 was first incubated with ATP as extract 2. After dialysis against TEL β buffer to remove ATP and phosphatase inhibitors, it was further incubated with dePi buffer for 30 min at 25°C (closed circles, dePi). The three extracts were applied to DE-52 columns of the same size (4 ml), respectively. The capping activity in fractions was measured as described in Mate-

Other Methods

The flow birefringence of F-actin solution was measured with an apparatus (Micro Flow Birefringence-MARK II; Wakenyaku Co., Japan) under a velocity gradient of 186/s. For convenience, the flow birefringence was represented as an arbitrary unit ($\Delta/2$ degree). 1 mg/ml of muscle and *Physarum* F-actin solutions showed $\Delta/2$ of 38° under our experimental conditions. This value corresponded to the flow birefringence of 1.2×10^{-5} . SDS-PAGE was performed according to the method of Laemmli (15) using a 5% concentration gel and a 12.5% separation gel. SDS-PAGE gels were stained with Coomassie brilliant blue or silver stained. The following marker proteins were used: phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,000), α -lactalbumin (M_r 14,000). Immunoblotting was performed using anti-fragmin antibodies according to the method as described (27). Protein concentration was determined using bovine serum albumin as a standard by the biuret reaction (7). In the case of *Physarum* actin, the absorbance at 540 nm was assumed to be 0.068 at 1 mg/ml.

Results

Reversible and Ca²⁺-dependent Inactivation of Fragmin-Actin Complex in Crude Extract

Actin regulatory proteins extracted from *Physarum* plasmodium were fractionated by a DEAE-cellulose column chromatography as shown in the previous paper (6). We found that the actin regulatory proteins including fragmin (peak 1 in Fig. 1 A), fragmin 60 and profilin, were eluted at the KCl concentration up to 100 mM, while the complexes of these proteins with G-actin were eluted at around 160 mM KCl (peak 2 in Fig. 1 A). From the results of gel filtration (AcA 44 column) and hydroxylapatite column chromatography of the fractions containing fragmin-actin complex, the capping activity in these fractions was mainly due to fragmin-actin complex (data not shown). This result was consistent with the data from gel filtration for purification of fragmin-actin complex (8).

Fig. 1 A shows the effect of ATP treatment in the absence of Ca²⁺ on the capping activity of fragmin or fragmin-actin complex in the crude extract. The capping activity in the complex fractions was observed when the extract was incubated without ATP (Fig. 1 A, peak 2 of open circles). Incubation of the crude extract with 5 mM ATP resulted in a marked decrease of the capping activity in the complexes fractions (Fig. 1 A, peak 2 of open squares). On the other hand, the capping activity of free fragmin fractions (Fig. 1 A, peak 1) was not affected by the ATP-treatment. The decrease in the capping activity of the complexes fractions was not due to dissociation of the fragmin-actin complex, because immunoblots of the complexes fractions with anti-fragmin antibodies revealed that the amounts of fragmin were nearly identical to those of the control (immunoblots in Fig. 1 A). Thus ATP was necessary for the inactivation of the fragmin-actin complex. When the ATP-treated extract was further incubated under conditions favorable for phosphatase reactions, the reduced capping activity of the com-

plexes and Methods. The presence of fragmin in fractions near peak 1 (free fragmin) and 2 (fragmin-actin complex) was shown by immunoblots of the fractions using anti-fragmin antibodies. The lower main band and the upper minor one represent fragmin and fragmin 60 (6), respectively. (B) Extract 4 was incubated in Pi buffer containing 0.4 mM CaCl₂, and 5 mM ATP. The volume of each fraction was 1.2 ml.

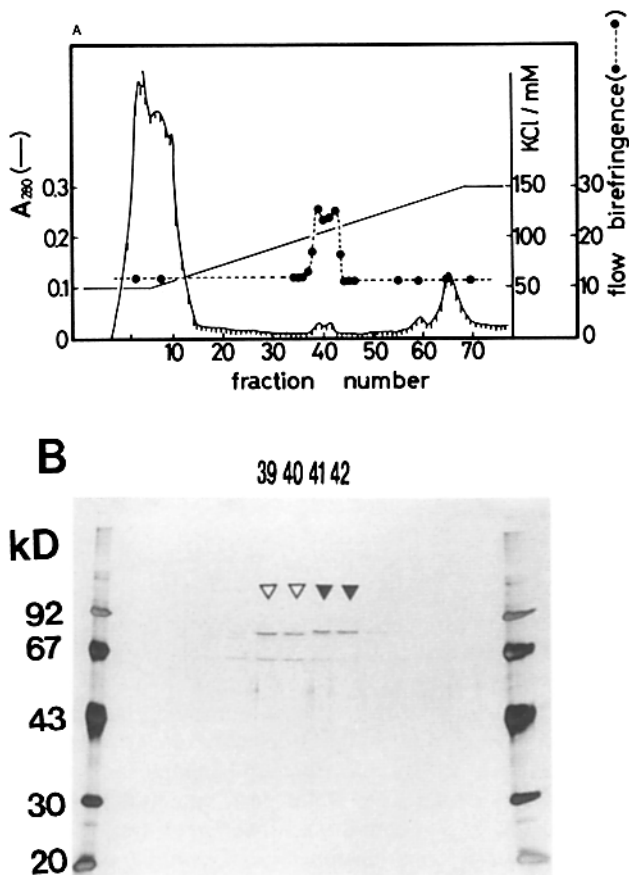


Figure 2. Purification of factors which inactivate fragmin-actin complex. (A) Mono Q column chromatography of the factors inactivating the complex. Closed circles and solid line represent the activity of the factors as described in Materials and Methods and the absorbance at 280 nm, respectively. (B) The silver-stained gel of SDS-PAGE of the fractions. The molecular masses of the factors were estimated to be 78 (open triangles), and 80 kD (closed triangles), respectively. Fraction volume was 1 ml.

plexes showed a partial restoration to its original activity (Fig. 1 A, peak 2 of closed circles). These results suggest that there exist some kinases and phosphatases in the crude extract, and the capping activity of the fragmin-actin complex may be regulated by phosphorylation and dephosphorylation of the complex.

When 0.4 mM Ca^{2+} instead of 1 mM EGTA was added to the crude extract, inactivation of the complex by ATP-treatment was not observed (peak 2 in Fig. 1 B). The peak of the capping activity observed at fraction number 20 was not due to the presence of free fragmin. Since free fragmin had bound to G-actin in the extract to form the fragmin-actin complex under these conditions, free fragmin was not detected by the immunoblots with anti-fragmin antibodies (peak 1 in Fig. 1 B). Therefore, only the complex was present in this case (peak 2 in Fig. 1 B). The peak at fraction number 20 would be due to the presence of another capping protein. In fact, the F-actin severing activity was not detected in this fraction.

Purification of Factors That Inactivate Fragmin-Actin Complex

We attempted to purify the factors that inactivate frag-

min-actin complex from the crude extract. The factors were fractionated by DEAE-cellulose column chromatography followed by chromatographies using DEAE-TOYOPEARL 650, Sephacryl S-300 HR, and Mono Q columns (FPLC). Fig. 2 A showed an elution profile of the factors by a Mono Q column chromatography, the final step for the purification. The activity of the factors that inactivated fragmin-actin complex (Fig. 2 A, closed circles) and the absorbance at 280

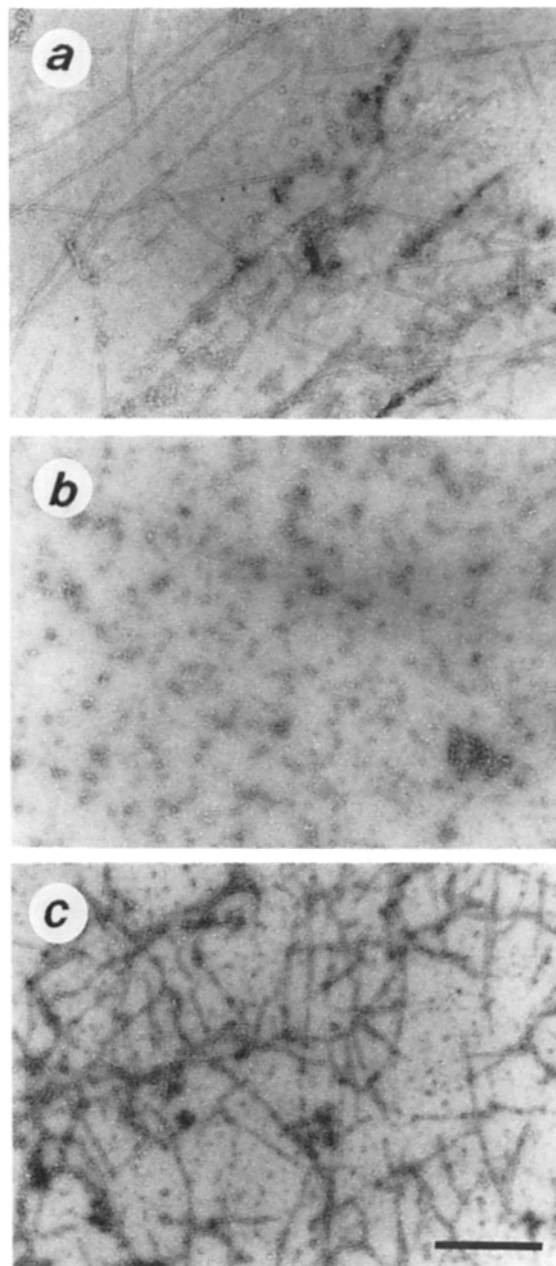


Figure 3. Electron micrographs of F-actin polymerized in the presence of inactivated complex. (a) *Physarum* G-actin (0.45 mg/ml) was polymerized in Pi buffer containing 1 mM EGTA and 5 mM ATP for 30 min at room temperature (control). (b) G-actin was polymerized in the same solution in the presence of purified fragmin-actin complex (0.3 mg/ml). (c) The complex was incubated with the factors in Pi buffer containing 1 mM EGTA and 5 mM ATP for 30 min at 25°C, and then G-actin was added to the solution. Bar, 0.5 μm .

nm (Fig. 2 A, *solid line*) showed two peaks at the fraction numbers 39–41. From the SDS-PAGE, the molecular masses of these two factors were estimated to be 78 and 80 kD, respectively (Fig. 2 B). One additional band (M_r 67,000) was slightly visible. However, we consider that this protein is not responsible for inactivation of fragmin–actin complex because, in our preliminary experiments, the position at which this protein was eluted from Butyl-TOYOPEARL or DEAE-TOYOPEARL columns (FPLC) did not correspond to that of inactivation activity. Both factors inactivated fragmin–actin complex in both the presence and absence of Ca^{2+} , and their Ca^{2+} -insensitive activity had been observed after the first step of chromatography for the purification. Therefore we used the fractions containing both factors as purified factors for the following experiments.

Inactivation of fragmin–actin complex by the purified factors was also confirmed by electron microscopy. When *Physarum* G-actin was polymerized in the presence of the complex, many short actin filaments were observed (Fig. 3 b). However, when it was polymerized in the presence of the complex which was incubated with the purified factors and ATP (Fig. 3 c), the long filaments were noted to be similar to that polymerized in the absence of the complex (Fig. 3 a).

Phosphorylation of Actin of Fragmin–Actin Complex by Factors That Inactivate Complex

ATP was necessary for the inactivation of the fragmin–actin complex, and the incubation suitable for phosphatase reactions caused restoration of the activity of inactivated complex (Fig. 1). To determine whether the factors phosphorylate the fragmin–actin complex, we carried out assays for phosphorylation of the complex by the purified factors.

Two kinds of fragmin–actin complexes were used, complex purified from crude extract and complex reconstituted from purified fragmin and G-actin. They were incubated with the purified factors in Pi buffer containing [γ - ^{32}P]ATP. After phosphorylation reaction the complexes were separated into fragmin and actin to determine which component, fragmin or actin, was phosphorylated, because fragmin has the same molecular weight as actin (Fig. 4 A). Fig. 4 B shows the autoradiography of the separated fragmin and actin. Only actin (Fig. 4 B, lanes 2 and 6) was phosphorylated in both purified and reconstituted complexes. No incorporation of ^{32}P into fragmin (Fig. 4 B, lanes 1 and 5) was observed. Actin alone was hardly phosphorylated (Fig. 4 B, lane 7). So we concluded that the factors are kinases which phosphorylate actin of the complexes. We termed these kinases “actin kinase.”

Inactivation of Fragmin–Actin Complex Is Induced by Phosphorylation of Complex's Actin

The time course of phosphorylation of the complex by actin kinase and that of the inactivation of the complex were monitored under the same conditions. Fig. 5 (*left*) demonstrates that the flow birefringence of F-actin (*closed circles*) increased with the increased incorporation of ^{32}P into actin of the purified complex (*open circles*). When the incorporation of ^{32}P into actin of the complex reached a maximum value at ~ 60 min, the flow birefringence reached the level equal to that of F-actin polymerized without the complex. Thus the complex was completely inactivated by the full phosphoryla-

tion. In the case of the reconstituted complex a similar relation was observed (Fig. 5 *right*). This decrease in the capping activity of the complexes was not due to proteolytic degradation of the complexes, since no fragmented polypeptides were detected on the SDS-PAGE gels of the complexes after the incubation. Therefore, we concluded that phosphorylation of actin of fragmin–actin complex induced the inactivation of its capping activity. Phosphate incorporated into 1 mol of actin of the reconstituted complex was 0.88, 0.95, and 1.11 mol in three experiments. The average value of incorporated phosphate was 0.98 mol of Pi/mol of actin. This value indicates that 1 mol of phosphate was incorporated into one mole of actin of the complex.

Elongation of Short F-Actin Capped with Fragmin–Actin Complex by Actin Kinase

When G-actin is polymerized in the presence of fragmin–actin complex, the barbed end of the short F-actin is capped with the complex. To examine the ability of actin kinase to inactivate the complex that caps the F-actin, we performed the following experiments. *Physarum* G-actin (1 mg/ml) was polymerized in the presence of fragmin–actin complex (0.2 mg/ml) to produce the capped short F-actin. After actin kinase was added to the short F-actin solution, we measured the flow birefringence of the solution. The flow birefringence began to increase immediately after the addition of actin kinase, and reached the level of F-actin polymerized without the complex after 6 hr (*+ kinase* in Fig. 6). This demonstrates that actin kinase also can inactivate the complex which caps F-actin. However, a prolonged incubation was required for inactivation of this complex, compared with

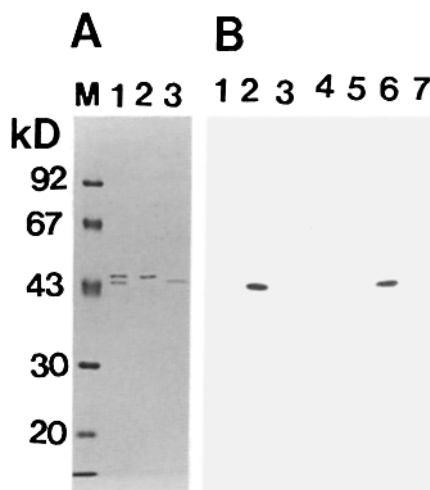


Figure 4. Phosphorylation of fragmin–actin complex by factors that inactivate fragmin–actin complex. (A) Lane 1, SDS-PAGE of purified fragmin–actin complex. Upper band, fragmin; lower one, actin. Lane 2, fragmin; lane 3, actin. Fragmin and actin were separated from the complex as described in the text. The proteins were stained with Coomassie brilliant blue. (B) Autoradiography of fragmin and actin. Lanes 1 and 2, fragmin and actin from purified complex which was phosphorylated with the factors; lanes 3 and 4, fragmin and actin from purified complex that was incubated in the absence of the factors; lanes 5 and 6, fragmin and actin from reconstituted complex that was phosphorylated with the factors; lane 7, actin incubated with the factors.

the inactivation of the complex alone. In the latter case, the same amount of actin kinase caused the complete inactivation of the complex within 10 min under the same conditions.

Discussion

We demonstrated in the present study that phosphorylation of fragmin-actin complex by actin kinase induced inactivation of its capping activity. The ATP-dependent inactivation of fragmin-actin complex in the crude extract was observed in the absence of Ca^{2+} , but not in the presence (Fig. 1). However, we found that purified actin kinase inactivated the complex in both the presence and absence of Ca^{2+} . So it is considered that an unknown factor(s) in the extract may give the Ca^{2+} sensitivity to actin kinase. There is another possibility that phosphatase(s) which dephosphorylates the actin phosphorylated by actin kinase has this Ca^{2+} sensitivity. As shown in Fig. 1 B, 0.4 mM CaCl_2 was added to the crude extract to examine the effect of Ca^{2+} on the inactivation of the complex. This Ca^{2+} concentration appeared to be somewhat higher than the physiological concentration of Ca^{2+} . However, we found that there was an unknown factor(s) which reduced Ca^{2+} concentration in the crude extract. Further experiments are necessary to determine the threshold concentration of Ca^{2+} in the crude extract.

The molecular mass of two kinds of actin kinase were estimated to be 78 and 80 kD by SDS-PAGE (Fig. 2 B), and actin kinase was eluted at around 110 kD by gel filtration (Materials and Methods). Further analysis of properties of actin kinase including the primary structures will allow us to clarify the similarity and/or difference between actin kinase and other protein kinases including protein kinase C (20).

Maruta et al. (19) isolated a protein complex consisting of Cap 42 (a) and Cap 42 (b) from *Physarum plasmodium*. They showed that the complex capped the barbed end of F-actin. They also reported that Cap 42 (b) of the complex could be phosphorylated by an endogenous kinase (Cap 42 (b) kinase), and the capping activity of the complex was regulated by the phosphorylation (17). Ampe and Vandekerckhove analyzed the amino acid sequences of fragmin and Cap 42 (a) and (b) (1). They reported that Cap 42 (b) was *Physarum* actin from which some terminal peptide is lost, and Cap 42 (a) was most

likely to be fragmin. However, our experiments on phosphorylation of fragmin-actin complex were clearly different from the case of Cap 42 (a + b) in the following points. (a) Phosphorylated Cap 42 (a + b) capped F-actin in the presence of Ca^{2+} , but not in the absence of Ca^{2+} (17). In the case of the fragmin-actin complex, the phosphorylated complex showed no activity in the absence and presence of Ca^{2+} . (b) Cap 42 (b) isolated from the complex possessed the capping activity, and could be phosphorylated (18). We would like to point out that at least the native state of *Physarum* actin was hardly phosphorylated (Fig. 4, lane 7). (c) No dephosphorylation of Cap 42 (b) was observed even in the crude extract (19). On the other hand, inactivation of fragmin-actin complex was reversible in the crude extract (Fig. 1 A). This result raises the possibility of reversible phosphorylation-dephosphorylation of fragmin-actin complex. (d) Although the molecular mass of Cap 42 (b) kinase was ~ 90 kD in the initial step of the purification (19), the molecular mass of purified Cap 42 (b) kinase was estimated to be 35 kD (18), which was less than half of that of actin kinase (~ 110 kD). All the results clearly distinguish the phosphorylation of fragmin-actin complex from that of Cap 42 (a + b). Particularly, it is impossible to explain the result that fully phosphorylated Cap 42 (a + b) possessed the capping activity equal to unphosphorylated Cap 42 (a + b) in the presence of Ca^{2+} (17) by the data from the experiments we performed. In this study we used the complex that was constituted from purified fragmin and actin in addition to the complex purified from the crude extract. Actin kinase phosphorylated and inactivated both the purified and the reconstituted complexes in the same way (Fig. 5). These results clearly demonstrate that intact fragmin-actin complex was phosphorylated and inactivated by actin kinase. It should be reexamined whether Cap 42 (a + b) is able to acquire Ca^{2+} sensitivity on its capping activity by phosphorylation under native conditions.

As shown in Fig. 1, the activity of peak 2 decreased by the incubation of the crude extract with ATP. Our previous studies showed these fractions contained complexes of various actin regulatory proteins and G-actin (6). It is interesting to examine the effects on their functional properties of phosphorylation of complexes of actin with actin regulatory proteins other than fragmin. These lines of experiments are in progress in our laboratory.

We assessed the ability of actin kinase to inactivate fragmin-actin complex which capped the barbed end of short

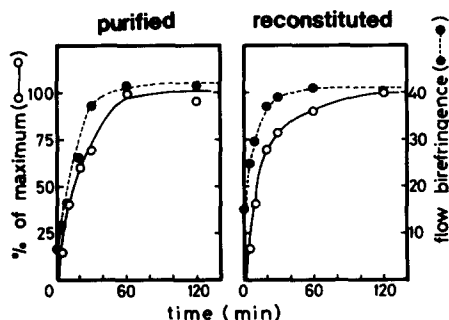


Figure 5. Phosphorylation and inactivation of fragmin-actin complex. Incorporation of ^{32}P i into actin of the complex (open circles) and inactivation of the complex (closed circles) were monitored under conditions described in Materials and Methods. Both purified complex (left) and reconstituted one (right) were used as fragmin-actin complex. The average value of 0.98 mol of P_i /mol of actin of the complex was obtained from three measurements.

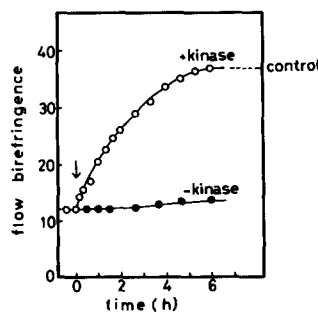


Figure 6. Elongation of short F-actin capped with fragmin-actin complex by actin kinase. Short F-actin capped with the complex was prepared by incubating *Physarum* G-actin (1 mg/ml) with reconstituted complex (0.2 mg/ml) in P_i buffer containing 1 mM EGTA and 5 mM ATP at 25°C for 15 min. At time indicated by an arrow, actin kinase (open circles) and buffer (closed circles) were added to the short F-actin solutions, respectively. The flow birefringence of the solutions was measured. The level of the flow birefringence of F-actin polymerized in the absence of the complex was indicated as control.

F-actin. When actin kinase and ATP were added to the short F-actin capped with the complex, the flow birefringence immediately began to increase and reached the level of that of F-actin polymerized in the absence of the fragmin-actin complex (Fig. 6). Such an increase in flow birefringence was never observed under conditions without ATP. These results showed that the actin kinase inactivated the complex on the barbed end of the short F-actin, and this brought about annealing of short F-actin. However, the inactivation of the complex that capped F-actin took a longer time than the inactivation of the complex alone. There may be an unknown factor(s) that accelerates inactivation of the complex on the barbed end of F-actin in vivo. In fact, fragmin-actin complex in the crude extract was inactivated within 10 min by addition of ATP (Fig. 1 A). Under these conditions involving high concentrations of Mg^{2+} , endogenous G-actin in the crude extract should be polymerized to form the short F-actin capped with the complex. Uyeda et al. observed a rapid transition of fragmented short F-actin into a bundle of long F-actin in *Physarum* flagellate (26).

In this study fragmin of the complex did not separate from actin even when actin of the complex was phosphorylated. However, we speculate that if phosphorylated complex is dephosphorylated, it becomes the nucleation site for actin polymerization at any part of the plasmodium. There may be another mechanism or unknown factors which dissociate fragmin and actin of the complex as PIP_2 dissociates the gelsolin-actin complex (13).

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