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Identification of actin kinase activity in purified fragmin–actin complex

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Actin kinase phosphorylates actin of fragmin–actin complex, resulting in the inactivation of the nucleation and capping activities of the complex. Fragmin–actin complex was prepared by a new purification procedure. Incubation with ATP caused inactivation of the purified complex and phosphorylation of actin of fragmin–actin complex. The detailed analysis of the complex by SDS-gel electrophoresis showed that actin kinase was co-purified with the fragmin–actin complex. Formation of such an association between actin kinase and substrate suggests that the kinase is localized on the fragmin–actin complex to efficiently regulate actin cytoskeletons.

Cytoskeleton; Actin filament; Phosphorylation; Actin regulatory protein

1. INTRODUCTION

It is generally accepted that actin-binding proteins play key roles in the dynamics of the actin cytoskeleton [1–3]. Fragmin purified from plasmodium of *Physarum polycephalum* binds G-actin and severs F-actin in the presence of μM amounts of Ca^{2+} [4]. The fragmin–actin complex, which is composed of fragmin and G-actin, promotes the nucleation step of actin polymerization because the complex can be a nucleus for the growth of actin filaments. This complex also binds to the barbed (fast growing) end of the actin filament and blocks association of G-actin and F-actin to it [5]. Therefore, the length of actin filaments polymerized in the presence of the complex is much shorter than in its absence.

Previous studies have shown that the actin of the fragmin–actin complex can be phosphorylated by actin kinase purified from *Physarum*, which abolishes the nucleation and capping activities of the complex [6]. Actin kinase phosphorylates fragmin–*Physarum* actin, fragmin 60–*Physarum* actin, and fragmin–muscle actin complexes in a highly specific manner [7–9]. The actin phosphorylation site of actin kinase resides within three consecutive threonines, Thr-201–203 [9]. It has been reported that, on the basis of X-ray analysis of muscle G-actin–DNase I complex, this site is located at the pointed end-domain of the actin molecule [10]. Biochemical studies have shown that fragmin caps the barbed ends of actin filaments [11], therefore inactivation of the fragmin–actin complex may be achieved by changes in charge or conformation of the pointed end-domain of the actin molecule.

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The present study shows that actin kinase binds tightly to the fragmin–actin complex. Such a tight binding suggests that actin kinase may be localized on the fragmin–actin complex to regulate the actin cytoskeletons.

2. EXPERIMENTAL

2.1. *Protein preparation*

Muscle G-actin was prepared as described [12]. Fragmin–actin complex was purified according to the previously described method [4] with a slight modification: concentration of proteins by ammonium sulfate precipitation before each column step was omitted to avoid loss of the actin kinase activity.

2.2. *Assays for phosphorylation and inactivation of the fragmin–actin complex*

Fragmin–actin complex (0.2 mg/ml) was incubated with 0.2 mM [γ-^{32}P]ATP (3 μCi) in a solution (60 μl) containing 30 mM Tris-HCl, pH 8.2, 4 mM $MgCl_2$, 1 mM DTT, 5 mM NaF, 0.1 mM EGTA, 0.1 mg/ml leupeptin, at 25°C for 2 h. The sample buffer (60 μl) for SDS-PAGE was added to the incubated sample, and the mixture was subjected to SDS-PAGE, followed by autoradiography. For inactivation assays, fragmin–actin complex was incubated under the same conditions except without [γ-^{32}P]ATP. 40 μl of 2.5 mg/ml muscle G-actin were added to the incubated sample solution (60 μl). After incubation at 25°C for 6 min, the flow birefringence was measured according to the described method [6].

2.3. *Other methods*

SDS-PAGE was carried out according to the method of Laemmli [13]. The following marker proteins were used: phosphorylase *b* (M_r 94,000), bovine serum 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). Autoradiography was performed as described [6].

3. RESULTS

In previous studies the purified fragmin–actin complex was not phosphorylated following incubation with

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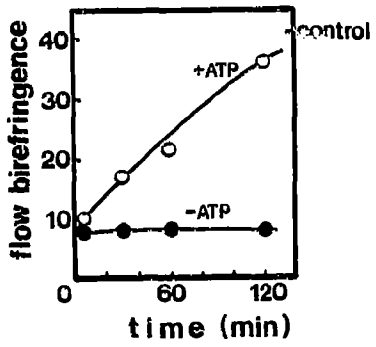

Fig. 1. Inactivation of fragmin-actin complex by incubation with ATP-containing solution. After fragmin-actin complex was incubated with ATP or buffer solutions, G-actin was added to the reaction medium, and polymerization of actin followed. The value of flow birefringence of F-actin solution conveniently represents the length of actin filaments.

ATP alone [6]. In the present work, fragmin-actin complex was purified by a new purification procedure in which ammonium sulfate precipitation for concentrating proteins was omitted. When G-actin was polymerized in the presence of the fragmin-actin complex, the length of actin filaments was very short (closed circles in Fig. 1), compared with that of control F-actin (control in Fig. 1). Incubation of fragmin-actin complex with ATP inactivated the capping activity of the complex, leading to an increase in the value of flow birefringence (open circles in Fig. 1). After incubation with ATP for 2 h the flow birefringence of F-actin solution containing the complex increased to close to control levels (Fig. 1). Thus, the fragmin-actin complex purified by the new method was inactivated by incubation with ATP alone.

We have demonstrated that the phosphorylation of actin of the fragmin-actin complex by actin kinase is responsible for inactivation of the complex [6,8,9]. Because there is a possibility that the inactivation of the complex incubated with ATP alone is due to phosphorylation of actin of the complex by actin kinase, the complex was incubated with [γ-^{32}P]ATP and subjected to SDS-PAGE, followed by autoradiography. The autoradiography of the incubated complex showed the phosphorylation of actin of the complex but not fragmin (Fig. 2). This result indicates that the fragmin-actin complex purified by the new methods contains the actin kinase activity.

To detect actin kinase in the fragmin-actin complex preparation, we carried out a detailed analysis of the SDS-PAGE of fractions from a DEAE-Toyopearl 650 S column. Free actin kinase was eluted from the DEAE-Toyopearl column at 100 mM KCl while fragmin-actin complex was eluted at 130 mM [6]. Actin kinase can be completely separated from the complex by this column chromatography. Fig. 3A shows an elution profile of fragmin-actin complex from the DEAE column. The Coomassie-stained gel of each fraction showed that the fragmin-actin complex was highly purified (Fig. 3B). Minor proteins in the complex fraction were detected by silver-staining of the same gel (Fig. 3C). A protein (indicated by an arrow) clearly co-eluted with the complex from the DEAE column. The molecular weight of this protein is about 80 kDa, and is consistent with that of actin kinase [6]. These results suggest that this protein is actin kinase.

4. DISCUSSION

Our previous studies showed that purified fragmin-actin complex was not phosphorylated by incubating with a solution containing MgATP. The complex purified by this new procedure, however, exhibited the actin kinase activity. In our preliminary experiments, the concentration step with ammonium sulfate was found to decrease the actin kinase activity to a great extent. Therefore, we omitted the concentration procedure to keep the high activity of this kinase.

Fragmin-actin complex was highly purified by chromatography using four kinds of columns: DEAE-cellulose, Sephacryl S-300, hydroxyapatite, and DEAE-Toyopearl. It is unlikely that the preparation of the highly purified complex contained free actin kinase because it should have been eluted from the DEAE-cellu-

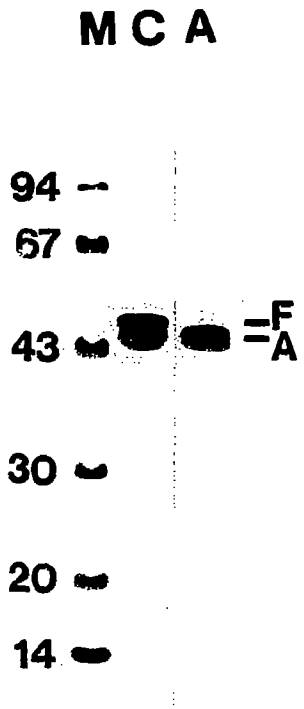

Fig. 2. Phosphorylation of actin of fragmin-actin complex by co-purified actin kinase. The purified complex was incubated with [γ-^{32}P]ATP, and then subjected to SDS-PAGE. Lane M, marker proteins; lane C, Coomassie-stained gel of the incubated complex; lane A, autoradiogram of the incubated complex. Molecular weight markers represent kDa.

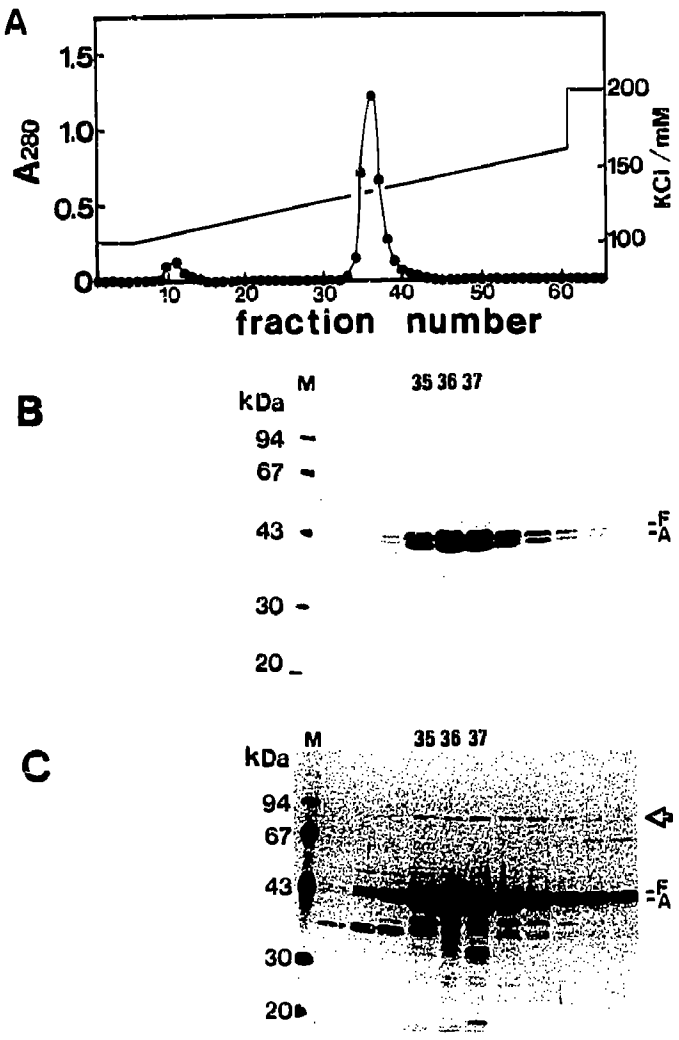

Fig. 3. Co-purification of actin kinase with fragmin–actin complex. (A) Elution profile of fragmin–actin complex from a DEAE-Toyopearl column. The complex was eluted from the DEAE column as described [6]. Protein concentration was monitored by absorbance at 280 nm. (B) Coomassie-stained gel of SDS-PAGE. Each fraction from the DEAE-column was subjected to SDS-PAGE, and then the gel was stained with Coomassie brilliant blue. F and A represent fragmin and actin, respectively. Numbers above the gel indicate fraction number. (C) Silver-stained gel of SDS-PAGE. The same gel as in B was silver-stained. Arrow shows a protein which co-eluted with the fragmin–actin complex. Note that this protein has the same molecular weight as actin kinase.

lose column at a concentration of KCl different from the fragmin–actin complex. The elution profile of the fragmin–actin complex from the DEAE–Toyopearl column showed that a protein with a molecular weight of 80 kDa co-eluted with fragmin–actin complex. This molecular weight is consistent with that of actin kinase. These results strongly suggest that this 80 kDa protein is actin kinase. Whether the actin kinase bound to the fragmin–actin complex is a different molecule to the free one is unknown. Another possibility is that free actin kinase gains the ability to bind the fragmin–actin complex by post-translational modification. It will be necessary to purify the bound actin kinase and characterize it in more detail to resolve this issue.

Actin kinase possesses a high specificity for protein substrates, as described [8,9]. It phosphorylates only actin of fragmin–actin, fragmin–muscle actin, and fragmin 60–actin complexes. This high specificity of actin kinase may contribute to a specific regulation of actin cytoskeleton. Moreover, the tight binding of actin kinase to the protein substrate, fragmin–actin complex, was detected during purification of the complex. Such association of actin kinase with the substrate suggests that the kinase efficiently regulates the actin cytoskeleton in a localized area of *Physarum* plasmodium. Other immunocytochemical studies have shown that some protein kinases are located on the cytoskeletal structure: type 3 protein kinase C on the focal contact [13], and myosin light chain kinase on the stress fibers [14,15]. A physiological role for such associations of protein kinases with actin cytoskeletons remains to be elucidated.

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