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journal or publication title	Biochemical and biophysical research communications
volume	390
number	1
page range	54-59
year	2009-09
権利	(C) 2009 Elsevier Inc.
URL	http://hdl.handle.net/2241/104039

doi: 10.1016/j.bbrc.2009.09.063

Usual and unusual biochemical properties of ADF/cofilin like-protein

Adf73p in ciliate *Tetrahymena thermophila*

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Abstract

ADF/cofilin is a well-conserved actin-modulating protein, which induces reorganization of the actin cytoskeleton by severing and depolymerizing F-actin. ADF/cofilin also binds to G-actin and inhibits nucleotide exchange, and hence, is supposed to regulate the nucleotide-bound state of the cellular G-actin pool cooperating with profilin, another well-conserved G-actin-binding protein that promotes nucleotide exchange. In this report, we investigated the biochemical properties of the ADF/cofilin-like protein Adf73p from ciliate *Tetrahymena thermophila*. Adf73p also binds to both G- and F-actin and severs and depolymerizes F-actin. Unlike canonical ADF/cofilin, however, Adf73p accelerates nucleotide exchange on actin and allows repolymerization of disassembled actin. These results suggest that the actin cytoskeleton of *T. thermophila* is regulated by Adf73p in a different way from those of mammals, plants, and yeasts.

Keywords:

ADF/cofilin

Tetrahymena

protist

nucleotide exchange

Introduction

Actin is a highly conserved cytoskeletal protein among eukaryotic cells [1]. Monomeric actin (G-actin) assembles into helical filaments (F-actin) and exerts various cellular functions such as cell motility, cytokinesis, endocytosis, and phagocytosis. The actin subunit has two distinct ends, and all the subunits align in the same orientation within the filament, which endows the filament with polarity. This polarity determines the directional movement of the myosin motor and enables the dynamic rearrangement of the actin cytoskeleton *in vivo*. At the barbed end (B-end), the association/dissociation of subunits is more frequent than at the pointed end (P-end). Moreover, the critical concentration (C_c) (the minimum concentration required for polymerization) of the B-end is lower than that of the P-end. Consequently, new subunits are incorporated into the B-end and dissociate from the P-end at steady state. In addition, nucleotides, especially ATP, bind to actin monomers with high affinity and stabilize the molecule. ATP on G-actin is hydrolyzed to ADP-Pi following its incorporation into filaments, and Pi is subsequently released from the subunits. Furthermore, ATP-G-actin has a higher affinity for the

B-end than ADP-G-actin. Therefore, ATP-G-actin becomes associated with the filament at the B-end and ADP-G-actin dissociates from the P-end at steady state, which is called the "F-actin treadmill".

In vivo, many actin-binding proteins (ABP) control actin dynamics. Profilin, a highly conserved ABP, binds to G-actin and accelerates nucleotide exchange [2]. Profilin-G-actin complexes are incorporated into the B-ends of F-actin as G-actin alone, but not into the P-ends. An actin-nucleator formin, which is regulated by Rho signaling, facilitates the incorporation of profilin-G-actin complexes, and hence, mediates the rapid growth of filaments from the B-ends [3]. This system is important for the construction of long, polarized F-actin structures such as cables. Furthermore, the Arp2/3-complex and its activator WASP promote formation of an actin-meshwork from the profilin-G-actin pool under Rho signaling [3]. Meanwhile, disassembly of the actin cytoskeleton is essential for its rearrangement upon cell signaling. ADF/cofilin, one of the well-conserved ABP among eukaryotes, severs F-actin and induces monomer dissociation from the P-end [4]. The ADF/cofilin-mediated severing of F-actin also produces free (non-capped) new ends from which

G-actin can polymerize. Thus, ADF/cofilin is supposed to accelerate the rate of actin turnover by promoting both the polymerization and depolymerization of F-actin *in vivo*. In addition, ADF/cofilin binds preferentially to ADP-G-actin and inhibits nucleotide exchange.

Alveolata is a diverse monophyletic group of protists and mainly consists of three groups, ciliates, dinoflagellates, and apicomplexans [5]. Recently, the unique properties of apicomplexans actins with regard to their specialized gliding motility have been reported [6]. A biochemical study of *Plasmodium falciparum* actin revealed that its filaments are unstable and very short [7]. Moreover, F-actin from *Toxoplasma gondii* is also short although its Cc is relatively lower than those of actins in mammals, yeasts, and plants [8]. Interestingly, *Plasmodium* ADF/cofilin-like protein ADF1 shows unusual biochemical properties; it binds only to G-actin and stimulates nucleotide exchange [9]. Therefore, ADF1 may be involved in the rearrangement of the actin cytoskeleton during F-actin assembly rather than disassembly.

In the free-living ciliate *Tetrahymena*, the actin cytoskeleton is found around phagosomes and in the cleavage furrow during cytokinesis

[10]. However, only a few actin-modulating proteins have been studied in this organism [11,12,13]. Recently, the genome sequences of *T. thermophila* were completely determined [14]. We found a single gene encoding an ADF/cofilin-like protein, *ADF73*, in the genome database and here report the biochemical properties of Adf73p.

Materials and methods

Gene cloning. A DNA fragment encoding *ADF73* was amplified from a cDNA library by PCR using primers, 5'-GGGAATTCCATATGGATATCGGCTTACAAG-3' and 5'-CCCTCGAGTCAATGGATCTTCTTTTCGAGTTCTTCGG-3', and was cloned into pT7Blue (Takara Bio Inc., Tokyo, Japan). The DNA sequence of the insert was confirmed by sequencing using an ABI 377 DNA Sequencer (Applied Biosystems Japan Ltd., Tokyo, Japan).

Expression and purification of Adf73p. *ADF73* contains six TAA codons in its open reading frame. TAA corresponds to a glutamine residue and is a codon unique to *Tetrahymena*. For bacterial expression, all TAA codons in *ADF73* were substituted to CAA codons using the pALTER-Ex1 system (Promega KK, Tokyo, Japan). The modified *ADF73* (*ADF73M*) was inserted into pGEX4T-1 (GE Healthcare, UK, Ltd.) to yield pGEX4T-1-*ADF73M*. BL-21 *E. coli* was transformed with pGEX4T-1-*ADF73M* and grown in LB medium to an OD₆₀₀ of 0.4-0.8 at 37°C, and then protein expression was induced with 1 mM IPTG for 5 h.

The cells were collected and washed with buffer A (50 mM Tris-HCl (pH 7.5) and 1 mM EDTA), and resuspended in buffer A containing 1% Triton X-100, 1 mM DTT, and protease inhibitor mix (0.5 µg/ml leupeptin, 0.02 mM TLCK, 1 mM PMSF, and 1 µg/ml pepstatin A). The cells were lysed by sonication on ice. The lysate was clarified by centrifugation and incubated with Glutathione Sepharose 4B beads (GE Healthcare) overnight at 4°C. After being washed with buffer A, the beads were treated with thrombin (5 U/ml) in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2.5 mM CaCl₂ for 6 h at 25°C. The bead suspension was centrifuged, and the supernatant containing Adf73p separated from GST was further incubated with benzamidine beads (GE Healthcare) to remove thrombin.

Preparation of actin. Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt [15] and purified by gel filtration using the HILoad 16/60 Superdex 200 pg column (GE Healthcare) and G-buffer (5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂, 0.2 mM ATP, and 0.1 mM DTT). Purified G-actin was quickly frozen in liquid nitrogen and stored at -80°C. Pyrene-actin was prepared according

to the method of Kouyama and Mihashi [16].

Negative staining. F-actin (4 μM in monomer) was incubated in the absence or presence of 0.8 μM Adf73p or 0.8 μM XAC [17] for 2 min at 25°C in F-buffer (G-buffer plus 2 mM MgCl_2 , 50 mM KCl, and 1 mM ATP). After being diluted 10 times in F-buffer, the sample was mounted on the carbon-coated grid for 2 min and then negatively stained with 2% uranyl acetate for 30 sec and dried. Observation was performed under a transmission electron microscope TEM-1010 (JEOL Ltd., Tokyo, Japan).

Severing activity. F-actin (5 μM) was mixed with various amount of XAC or Adf73p for 1 min at 25°C in F-buffer. The sample was diluted to one tenth in G-buffer with pyrene-G-actin (1 μM , 11% pyrene labeled) in a cuvette and the polymerization was initiated by adding a tenth volume of G-buffer containing 500 mM KCl, 20 mM MgCl_2 and 20 mM ATP. The change in fluorescence intensity (excitation: 365 nm; emission: 407 nm) was monitored using a fluorometer RF-5300PC (SHIMADZU CORPORATION, Kyoto, Japan) at 25°C.

Cosedimentation assay. After 4 μ M actin had been polymerized in a neutral buffer (5 mM MES (pH 6.9), 2 mM $MgCl_2$, 100 mM KCl, and 2 mM ATP) or a basic buffer (5 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 100 mM KCl, and 2 mM ATP), various amounts of Adf73p were mixed for 2 h at 25°C. The samples were centrifuged at $100,000 \times g$ for 30 min. Supernatants and pellets were subjected to SDS-PAGE, and gels were stained by Coomassie Brilliant Blue (CBB). Gelsolin was purchased from Sigma-Aldrich Inc. (Tokyo, Japan).

G-actin-binding assay. G-actin (2 μ M) was mixed with various amounts of Adf73p or XAC in 5 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$, 0.2 mM ATP (or ADP), 1 mM DTT, and 10% glycerol overnight at 4°C. The samples were applied to 7.5% gel SuperSep (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and electrophoresed for 6-8 h at 7.5 mA and 4°C. The gels were stained by CBB.

Nucleotide exchange assay.

G-actin was incubated with Dowex 1-X8 (Dow Chemical Co., Michigan, USA.) in 2 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂ and 0.1 mM DTT for 5 min on ice. After centrifugation, the supernatant was incubated with 0.2 mM 1,N⁶-ethenoadenosine 5'-diphosphate (ϵ -ADP) for 4 h on ice. Free ϵ -ADP was removed with Dowex 1-X8. Various amount of XAC or Adf73p was added to the solution. After incubation for 10 min at 25°C, 0.2 mM ADP was applied. The change in fluorescence intensity (excitation: 360 nm; emission: 410 nm) was monitored using a fluorometer RF-5300PC at 25°C.

Results

ADF73 belongs to the ADF/cofilin-family

We found that there was a single ADF/cofilin-like gene in the *T. thermophila* genome database (TGD). After cloning it from the cDNA library and sequencing it, it was verified that *ADF73* consists of a single exon of 408 bp encoding 135 amino acid residues (DDBJ accession number, **AB511999**). The activity of metazoan ADF/cofilin is negatively regulated by the phosphorylation of Ser3 and that LIM kinase and slingshot phosphatase phosphorylates and dephosphorylates ADF/cofilin, respectively [18]. However, *ADF73* does not possess the corresponding Ser residue. Moreover, no genes encoding LIM kinase and slingshot phosphatase were found in TGD. Therefore, the activity of Adf73p may not be controlled by phosphorylation unlike that of metazoan ADF/cofilin.

Adf73p severs F-actin

We first investigated the F-actin severing activity of Adf73p by observing its effect on the length distribution of F-actin. F-actin was incubated with Adf73p or *Xenopus* ADF/cofilin XAC, and the filaments

were visualized under TEM and photographed (Fig. 1A). We categorized the observed filaments into three groups according to the number of ends found (0, 1, or 2) on an EM film: short filaments tended to belong to the 2-end group (Fig. 1B). Actually a large number of F-actin in the control belonged to the 0-end (45.2%) or 1-end group (42%). On the other hand, in the presence of Adf73p, the population of the 2-end group was increased to 64.4% while the 0-end group was decreased to 1.5%, suggesting that the number of short filaments was increased. The effect of Adf73p was comparable to that of XAC (2-end group: 85.9%; 0-end group: 1%). Thus, Adf73p may sever F-actin and produce short F-actin as well as XAC.

Next, to evaluate the severing activity of Adf73p in biochemistry, assembly of pyrene-G-actin was monitored in the presence of F-actin seeds (Fig. 1C). The intensity of pyrene-actin increases when it polymerizes [16]. The intensity was increased quickly in the presence of XAC as compared with actin alone, suggesting that it stoichiometrically accelerates actin polymerization by increasing the number of filaments. Adf73p also produced the quick increase of the intensity. Taking together, Adf73p severs F-actin and increases the number of new filaments ends where

pyrene-G-actin can associate, and hence, promotes net actin assembly.

Actin-depolymerization activity of Adf73p

Canonical ADF/cofilins depolymerize F-actin more effectively under basic conditions [4]. This may be derived from a pH-dependent conformational change in the actin molecule that alters the binding mode of ADF/cofilin to actin [19]. We assessed the F-actin depolymerization activity of Adf73p at both pH 6.9 and pH 8.0 using a cosedimentation assay. Unexpectedly, the amount of actin in the supernatant was not increased by incubation with Adf73p under either pH condition (Fig. 2A), suggesting that Adf73p did not affect the amount of unpolymerized actin. Meanwhile, the association of Adf73p with F-actin was verified by its cosedimentation with F-actin (actin in pellets) under both pH conditions although binding was less effective at pH 8.0. Thus, Adf73p appeared to bind to F-actin but not depolymerize it. Alternatively, it is also possible that Adf73p depolymerizes F-actin from the P-ends but that the monomers are allowed to repolymerize even in the presence of Adf73p. To test these possibilities, a cosedimentation assay was performed under conditions in which the

B-ends of F-actin were capped by gelsolin (Fig. 2B). The amount of actin in the supernatant was increased by Adf73p in a dose-dependent manner in the presence of gelsolin, suggesting that Adf73p was able to depolymerize F-actin from the P-end. Therefore, it is likely that the subunit association with the uncapped B-end is not suppressed even in the presence of Adf73p.

Adf73p binds preferentially to ADP-G-actin rather than to ATP-G-actin

ADF/cofilin generally binds more stably to ADP-G-actin than to ATP-G-actin and inhibits the nucleotide exchange on G-actin. We next examined the G-actin binding properties of Adf73p using native-PAGE (Fig. 3A). In native-PAGE, both G-actin and Adf73p produces a distinct, single band, and the mixture of ADP-G-actin and Adf73p produces a smear band, which probably corresponds to Adf73p-G-actin complex (Fig. 3A, left). On the other hand, only a small smear was detected when Adf73p was mixed with ATP-G-actin, suggesting that Adf73p hardly formed a complex with ATP-G-actin (Fig. 3A, right). The tendency was similar with XAC. Thus, Adf73p prefers ADP to ATP for G-actin binding, as does canonical ADF/cofilin.

Adf73p accelerates nucleotide exchange on G-actin

We measured the effect of Adf73p on the rate of nucleotide exchange of G-actin. The fluorescence intensities of ϵ -ADP and -ATP increase when they bind to G-actin [20]. Since the rate of nucleotide exchange of ϵ -ATP on actin was so fast even in the presence of Adf73p (data not shown), we decided to measure the effect of Adf73p on the rate of nucleotide exchange of ϵ -ADP on G-actin. The fluorescence intensity of ϵ -ADP-G-actin decreased exponentially during incubation with an excess amount of ADP, which represents that ϵ -ADP dissociates from G-actin (and exchanges with unlabeled ADP) according to first order kinetics (Fig. 3B, C). As previously reported for canonical ADF/cofilin [21], XAC reduced dissociation rate of ϵ -ADP about by 2-fold (Fig. 3B). In contrast, Adf73p stoichiometrically increased the dissociation rate up to about by 3-fold (Fig. 3C). Therefore, Adf73p was able to accelerate nucleotide exchange on G-actin in an opposite manner to canonical ADF/cofilin.

Discussion

Canonical ADF/cofilin depolymerizes ADP-actin subunits from the P-end of F-actin, forms a stable 1:1 complex with ADP-G-actin, and inhibits G-actin nucleotide exchange (Fig. 4). Thus, ADF/cofilin can produce a pool of ADP-G-actin from either G- or F-actin. In addition, ADP-actin subunits attach slowly to the B-ends and dissociate faster from the P-ends than ATP-actin subunits, and hence binding of ADF/cofilin to G-actin increases the Cc at the B-end. Consequently, the amount of unpolymerized actin (as ADP-G-actin) increases in the presence of ADF/cofilin *in vitro*. *In vivo* Cyclase-associated protein (CAP) and profilin facilitate recycling of ADP-G-actin into readily polymerizable ATP-G-actin [22,23].

In this study, we revealed the usual and unusual biochemical activities of the *T. thermophila* ADF/cofilin Adf73p (Table 1). Adf73p also binds to both G- and F-actin and severed F-actin. However, contrary to canonical ADF/cofilin, Adf73p increased the amount of unpolymerized actin only when the B-ends of F-actin were capped (Fig. 2). Moreover we found that Adf73p accelerated nucleotide exchange on actin although it

binds preferentially to ADP-G-actin rather than to ATP-G-actin similar to XAC (Fig. 3). Therefore, it is possible that the ADP on Adf73p-ADP-G-actin complex was rapidly exchanged to ATP in the cosedimentation assay since there was present 2 mM ATP (Fig. 2A). Here, we show a model for biochemical functions of Adf73p *in vitro* (Fig. 4). The regenerated ATP-G-actin by Adf73p may promptly dissociate from the complex and be incorporated into the B-end of filament. Alternatively, it is also possible that Adf73p-G-actin complex directly associates with the B-end as well as free G-actin. This idea is supported by the finding that Adf73p did not increase the amount of unpolymerized actin in a cosedimentation assay using ADP-F-actin (data not shown).

It is considered that the binding of canonical ADF/cofilin to G-actin closes the nucleotide-binding cleft and inhibits nucleotide exchange [24]. Meanwhile, profilin opens the cleft and accelerates the exchange when it binds to G-actin [25,26]. It has been demonstrated that ADF/cofilin and profilin compete to bind to G-actin [27]. Thus, these two proteins have opposite effects on G-actin nucleotide exchange although their binding regions probably overlap. It is possible that Adf73p may open the

nucleotide-binding cleft as profilin does and hence promote nucleotide exchange. In future it is important to study the conformation change of Adf73p-actin complex.

In *Tetrahymena*, actin polymerization is rapidly occurred in phagocytosis [28], and the F-actin around nascent phagosomes turns over rapidly (our unpublished data). However, knockdown of *Tetrahymena* profilin seems not to directly affect phagocytosis [29], even though profilin is generally involved in F-actin turnover. Thus, Adf73p possibly contributes to the rapid actin turnover required for phagocytosis by accelerating the regeneration of ATP-G-actin from aged ADP-F-actin redundantly with profilin. To verify this idea, now we are elucidating the intracellular activity of Adf73p.

Acknowledgements

We thank Drs. Shoichiro Ono and Sawako Yamashiro (Emory University) for their valuable comments and discussions. This work was supported by the Sumitomo Foundation.

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Figure Legends

Fig. 1. Adf73p severs F-actin. (A) Effect of Adf73p on the length of F-actin. F-actin (4 μM) was incubated with or without Adf73p or XAC (0.8 μM for each protein) and then negatively stained. Bar: 0.5 μm . (B) The filament length distributions were classified into three groups using the number of end(s) of a filament detected in an optical field: 2-ends (white) includes filaments for which both ends were detected, 1-end (black) were filaments lying across the field, and 0-end (grey) were filaments for which neither ends were detected. (C) Adf73p may increase the number of filament ends. Pyrene-G-actin (1 μM , 11% pyrene labeled) was added to the mixture of F-actin without or with Adf73p (left) or XAC (right) at 25°C. The amount of F-actin was recorded using the intensity of fluorescent signals.

Fig. 2. Cosedimentation of Adf73p with F-actin. F-actin (4 μM) was mixed with various amounts of Adf73p and incubated at pH 6.9 or pH 8.0 in the absence (A) or presence of 0.02 μM gelsolin (B) for 2 h at 25°C and then centrifuged at $100,000 \times g$ for 30 min. The supernatant (S) and pellet

(P) were subjected to SDS-PAGE. No band corresponding to gelsolin was detected since its concentration was too low.

Fig. 3. Adf73p binds to ADP- rather than ATP-G-actin. (A) Two micromolars of ADP- (left) or ATP-G-actin (right) were incubated with various amounts of Adf73p or XAC and then subjected to native-PAGE. (B) XAC inhibits nucleotide exchange on G-actin. ϵ -ADP-G-actin (0.5 μ M) was incubated with 0-1 μ M of XAC for 10 min at 25°C. The samples were supplemented with 0.2 mM ADP at 0 s, and the change in fluorescence intensity was monitored. (C) Adf73p accelerates nucleotide exchange on G-actin. ϵ -ADP-G-actin (0.2 μ M) was incubated with 0-2 μ M of Adf73p. Dissociation rate constants of ϵ -ADP were plotted against the concentration of XAC or Adf73p (right).

Fig. 4. *Tetrahymena* Adf73p may regulate actin turnover via a different biochemical property from canonical ADF/cofilin. Generally, ADF/cofilin rapidly dissociates ADP actin subunits from the P-end of F-actin and inhibits nucleotide exchange. CAP releases ADF/cofilin from its complex

with actin and enables profilin to interact with ADP-G-actin. Profilin binds to G-actin and stimulates nucleotide exchange. ATP-G-actin-profilin complex can be incorporated into the B-end of the filament but not the P-end. On the other hand, while Adf73p also promotes subunit dissociation from the P-end, it accelerates nucleotide exchange of G-actin contrary to canonical ADF/cofilin. Thus, it may be possible that Adf73p alone promotes actin turnover.

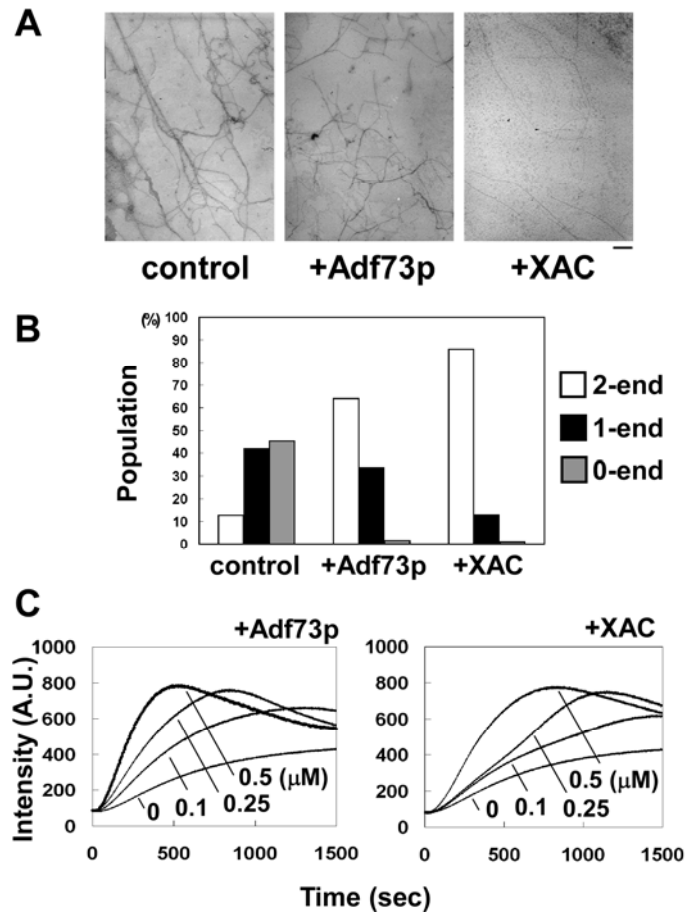


Fig. 1

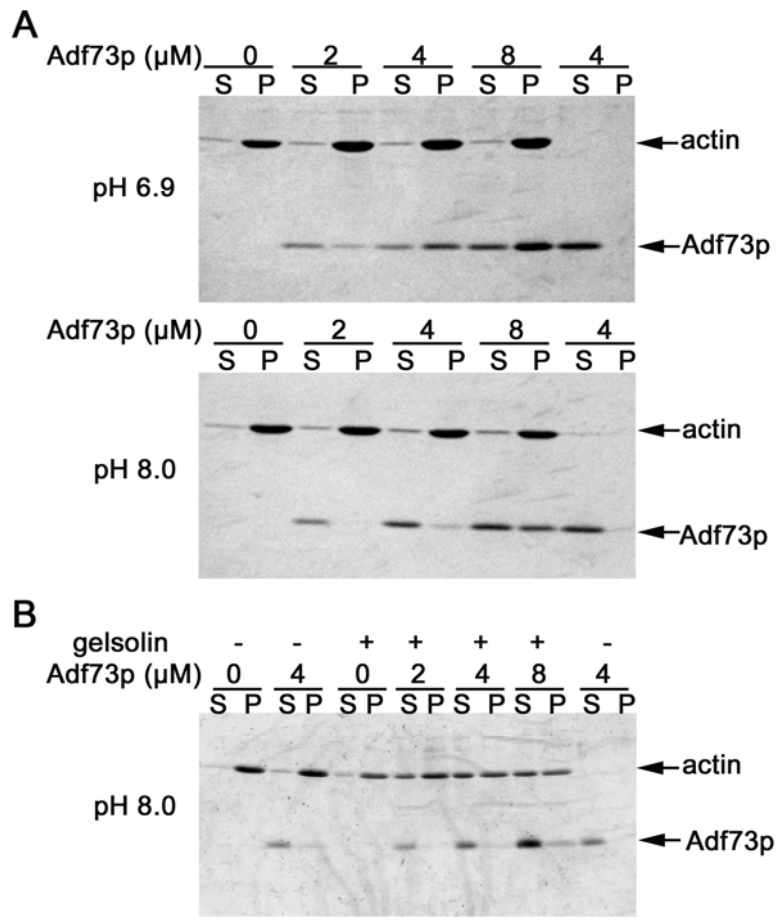


Fig. 2

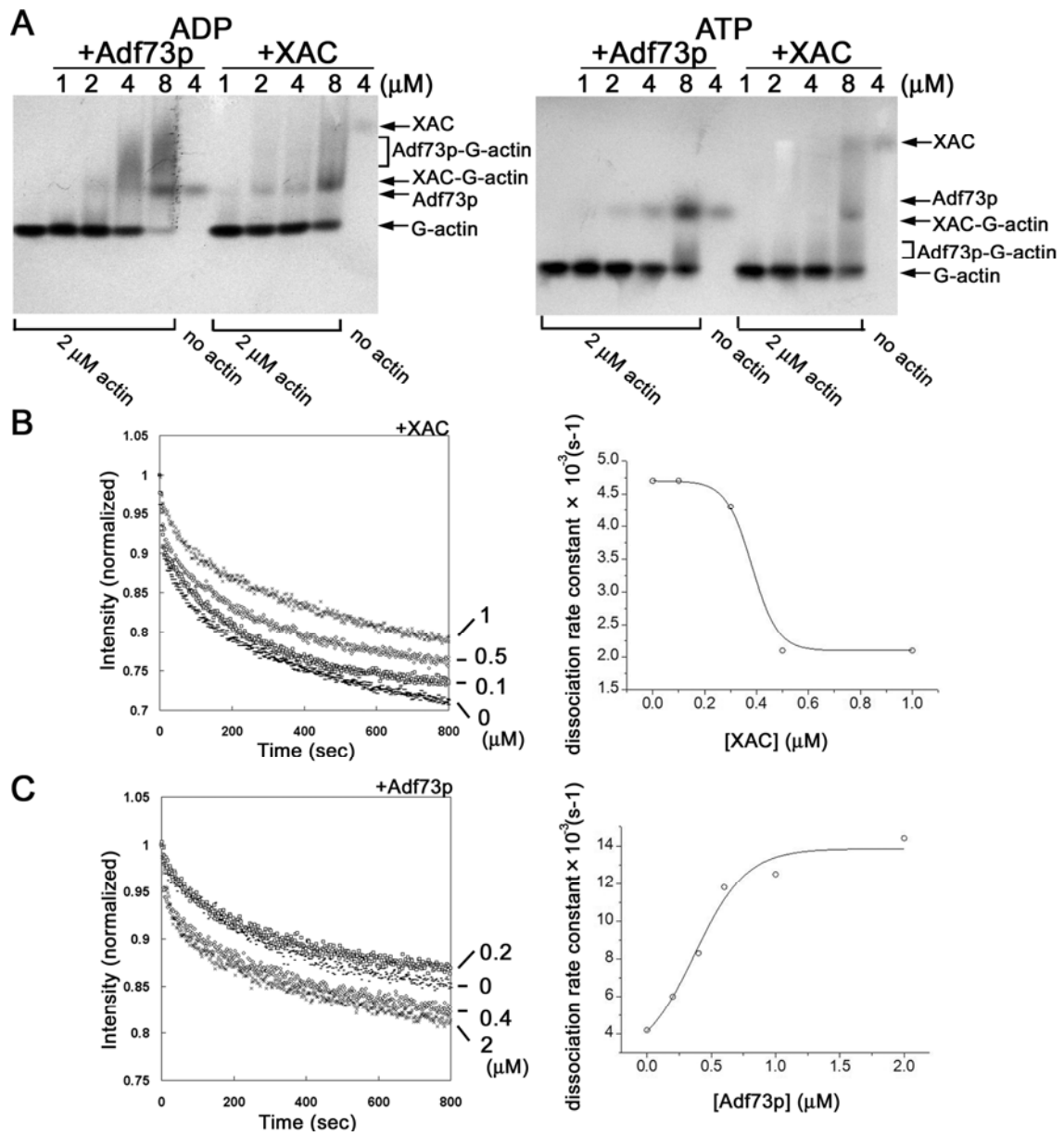


Fig. 3

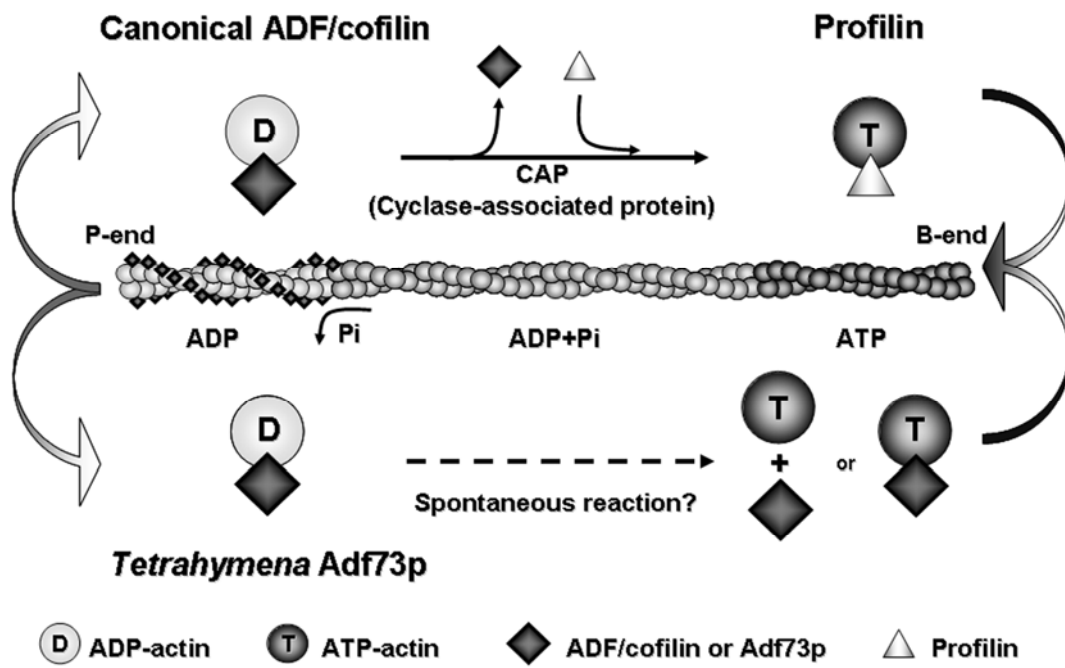


Fig. 4