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Enzymatic Form and Cytoskeletal Form of Bifunctional *Tetrahymena* 49kDa Protein Is Regulated by Phosphorylation

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ABSTRACT—*Tetrahymena* 49kDa protein functions as a citrate synthase (CS) and also assembles to 14-nm filament during cell mating. Bifunctional property of 49kDa protein is suggested to be maintained by the difference of post-translational modification(s). We have found that phosphorylation is present on all three isoforms of 49kDa protein. Dephosphorylation of citrate synthase type isoforms of 49kDa protein, composing pI 7.7 and 8.0 isoforms, reduced its enzymatic activity, shifting these isoforms to basic side. In a course of dephosphorylation, isoform of pI 8.4 appeared with pI 7.7 and 8.0 isoforms, which correspond to the isoforms of 14-nm filament assembling type. With this dephosphorylation, the citrate synthase type isoforms obtained the ability to assemble 14-nm filaments. We propose that enzyme form and cytoskeletal form of 49kDa protein were maintained simply by phosphorylation.

Key words: *Tetrahymena*, citrate synthase, phosphorylation, filament formation, bifunction

INTRODUCTION

In unicellular organism, ciliated protozoan *Tetrahymena*, 49kDa protein is identified as a mitochondrial citrate synthase (CS) and also as a cytoplasmic 14-nm filament forming protein (14FP) (Numata *et al*., 1985; Numata *et al*., 1991; Takagi *et al*., 1991; Numata, 1996). In vegetative growing state of the cell, 49kDa protein behaves as citrate synthase. However in cell conjugation, a sexual process of the cell, 49kDa protein forms 14-nm filament that is thought to play a role in selecting or locomoting gametic nuclei between conjugating cells.

In order to clarify this multiple role of CS in *Tetrahymena* cells, several approaches were done to characterize this protein. Using this protein nature that 14FP can be purified by centrifugation as filaments and CS can be purified from mitochondria, two different states of this protein were compared. CS purified from *Tetrahymena* mitochondria and 14FP purified by assembly and disassembly were identical in terms of molecular weight, antigenecity and enzymatic properties (Kojima *et al*., 1995). However, from two dimensional gel analyses, CS is comprised of two isoforms at pI 7.7 and 8.0, while 14FP has an additional pI 8.4 isoform (Kojima *et al*., 1997). Citrate synthase activity is present in the pI 7.7 and 8.0 isoforms from both CS and 14FP, although pI 8.4 isoform does not show the enzymatic activity (Kojima *et al*., 1997). Molecular biological approach revealed that a single gene encodes all of these isoforms and translated from single species of mRNA (Numata *et al*., 1996). In addition, *Escherichia coli* expressed recombinant 49kDa protein has citrate synthase activity and forms 14-nm filament in presence of 49kDa protein immunodepleated cytoplasm (Takeda *et al*., 1997). Therefore, we established a hypothesis that post-translational modification is responsible for polymorphism of these isoforms.

In this study, *in vivo* phosphorylation of 49kDa protein was detected by ^{32}P cell labeling technique. All three isoforms are phosphorylated. We show that *in vitro* phosphorylation and dephosphorylation of 49kDa protein are involved in regulating each enzymatic activity and filament-forming ability. Phosphorylation and dephosphorylation appears to be closely related to the regulation of bifunctional property of 49kDa protein.

MATERIALS AND METHODS

Cell culture

Cultivation of *Tetrahymena pyriformis* (strain W) was performed as described (Watanabe *et al*., 1994).

Purification of CS and 14FP

Purification of CS from *Tetrahymena* mitochondria was performed as described (Kojima *et al*., 1995). 14FP was purified from *Tetrahymena* acetone powder by the assembly and disassembly procedure (Numata and Watanabe, 1982; Takeda *et al*., 1995).

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Cell labeling and immunoprecipitation of 49kDa protein

Tetrahymena cells (0.5-1×10^6 cells/ml) were labeled *in vivo* with 0.3 mCi/ml [^{32}P] orthophosphate (PBS13, Amersham Pharmacia Biotech, Buckinghamshire, UK) in 10 mM Tris-HCl (pH 7.4) at 25°C. The cells were washed twice with 10 mM Tris-HCl (pH 7.4), and lysed in an equal volume of a lysis buffer (150 mM KCl, 2 mM $MgCl_2$, 0.2% SDS, 1% Nonidet P-40, 0.1 mM EGTA, 0.1 mM EDTA, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 2 μM microcystin-LR, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 0.1 mM N^{α}-tosyl-L-lysylchloromethyl ketone (TLCK), 0.1 mM pepstatin A, and 20 mM 3-(N-Morpholino) propanesulfonic acid, pH 7.4). The lysate was centrifuged at 10,000×g for 20 min. The supernatant was immunoprecipitated with anti-49kDa protein antibody-bound protein A sepharose (Pierce, Rockford, IL, USA) for 2 hr at 4°C. The beads were collected and washed six times with a half concentration of lysis buffer.

For dephosphorylation, the labeled cells were lysed in lysis buffer without NaF, Na_3VO_4 and microcystin-LR, and then centrifuged at 10,000×g for 20 min. The 49kDa protein was immunoprecipitated as described above. The precipitate was incubated with 1 unit of protein phosphatase 2A catalytic subunit (Wako, Osaka, Japan) at 37°C for 40 min.

***In vitro* dephosphorylation of purified CS**

Purified CS (1–2 mg/ml) were dialyzed against 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM oxaloacetic acid, 20% glycerol, 1 mM PMSF, 5 μg/ml leupeptin, 0.1 mM TLCK, and 0.1 mM pepstatin A. The dialyzed CS was incubated with 2 units of protein phosphatase 2A and 20 units of alkaline phosphatase catalytic subunit (Sigma-Aldrich, St. Louis, MI, USA) at 30°C for 2 hr, and then boosted with same phosphates for another 2 hr or 10 hr. The reaction was immediately stopped by addition of 2 μM microcystin-LR.

To exclude the phosphatase and to re-purify CS, the dephosphorylated CS was dialyzed against 5 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (pH 6.6) containing 0.5 mM oxaloacetic acid and 10% glycerol, and then applied to a Mono S column (Amersham Pharmacia Biotech). The fractions containing CS were dialyzed against 5 mM MES (pH 6.6) containing 50 mM KCl.

Filament assembly assay

Purified dephosphorylated CS, native CS, and the native 14FP were concentrated to 1–2 mg/ml. Polymerization procedure was performed as described (Numata and Watanabe, 1982; Takeda *et al*., 1995). Polymerized samples were negatively stained with 3% aqueous uranyl acetate and observed with a JEOL 100CXII electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Measurement for citrate synthase activity

Citrate synthase activity was measured according to the DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) method (Srere *et al*., 1963; Kojima *et al*., 1995). Briefly, the enzymatic activity was measured at 37°C in a reaction mixture (20 mM Tris/HCl/pH 7.75 containing 1 mM EDTA, 150 mM KCl, 50 M acetyl-CoA, 100 μM oxaloacetic acid and 100 μM DTNB). The reaction was started by adding 5 μl of each sample to 495 μl of reaction mixture and the increase in absorption at 412 nm per min was measured.

Two-dimensional gel electrophoresis (2-DE)

Preparation of electrophoresis samples and 2-DE were performed as described (Kojima *et al*., 1997). Proteins in gel were silver stained, and autoradiography of the gel was analyzed by a Bio image analyzer (BAS 5,000; Fujifilm, Tokyo, Japan).

Immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis were performed according to the methods of Laemmli (1970) and Towbin *et al.* (1977) with some modification (Edamatsu *et al*., 1991), respectively. The anti-14FP and anti-CS polyclonal antibodies were the same as those used in previously (Numata and Watanabe, 1982; Kojima *et al*., 1995).

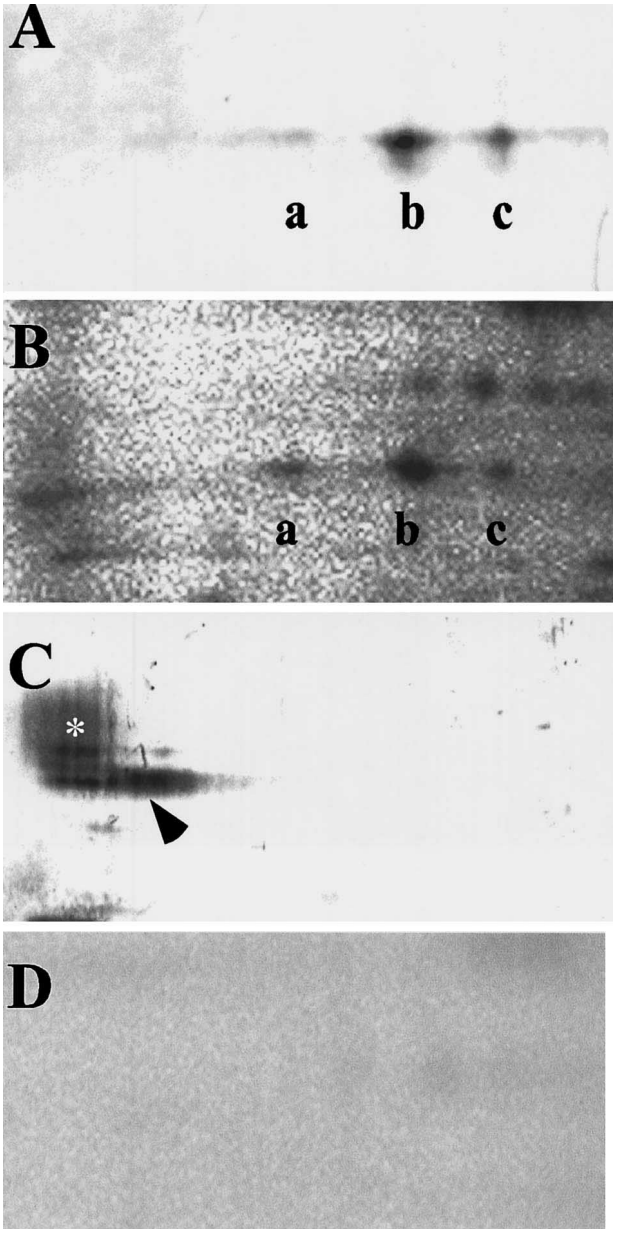

Fig. 1. Isoforms of 49kDa protein are phosphorylated. The ^{32}P-labeled cell lysate were immunoprecipitated with anti-49kDa protein antibody. The immunoprecipitate was subjected to 2-DE (A, B). The immunoprecipitate was dephosphorylated with protein phosphatase 2A, followed by 2-DE (C, D). The gels were silver stained (A, C) and the autoradiographic images of the gels were analyzed using a Bio image analyzer (B, D). The acid end of the gel is to the right. In (A) and (B), "a", "b", and "c" denote the pI 8.4, 8.0 and 7.7 isoforms. The arrowhead in (C) indicates the pI 8.8–9.0 isoform and the broad staining on upper left of this spot is the phosphatase (asterisk), which was removed by column chromatography as presented in Fig. 3C.

RESULTS

The isoforms of 49kDa protein are phosphorylated

The phosphorylation of isoforms was examined by labeling living *Tetrahymena* cells with ^{32}P orthophosphate, followed by immunoprecipitation of 49kDa protein. All three isoforms were phosphorylated, indicated by ^{32}P labeling (Fig. 1A, B). Dephosphorylation of 49kDa protein led all three isoforms to shift to a comet-shaped new pI 8.8–9.0 spot which was not labeled (Fig. 1C, D), suggesting that polymorphism of these isoforms are due to the phosphorylation.

Dephosphorylation decreases citrate synthase activity of the isoforms

In order to see the role of phosphorylation of these isoforms, we investigated the relation between phosphorylation of isoforms and citrate synthase activity. CS was dephosphorylated with protein phosphatase 2A and alkaline phosphatase. Enzymatic activity of CS decreased to nearly 30% of its initial activity after 3–4 hr, and was lost completely at 12 hr while the mock treated CS maintained its enzymatic activity to 95% at 4 hr and about a half at 12 hr of initial activity (Fig. 2). Previous study showed that the pI 7.7 and 8.0 isoforms have citrate synthase activity, but the pI 8.4 isoform does not (Kojima *et al*., 1997). Since the dephosphorylation leads to a shift of isoforms to the basic side (Fig. 1C) and decreased the citrate synthase activity (Fig. 2), it is likely that isoform on basic side is less phosphorylated and having lower citrate synthase activity.

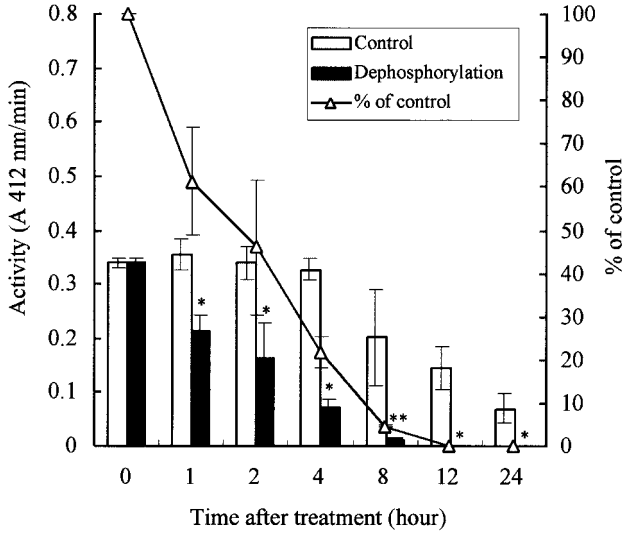

Fig. 2. Dephosphorylation reduces citrate synthase activity of 49kDa protein. Purified CS was treated with protein phosphatase 2A and alkaline phosphatase as described in **MATERIALS AND METHODS**. This treatment was boosted twice at 1 and 2hr after incubation. Citrate synthase activity was measured at 0, 1, 2, 4, 8, 12, and 24 hr after the addition of phosphatase. As a control, the buffer was added to reaction instead of phosphatase and subjected to same reaction condition. Enzymatic activities are shown as elevations of absorbance at 412 nm for 1 min (left axis, bar chart). Relative enzymatic activities are also represented as percentages of activities compared to control reaction at each time points (right axis, line graph). *p<0.01; **p<0.05, comparing treated samples to respective controls.

Dephosphorylated CS forms filaments *in vitro*

The 14FP, filament forming state of 49kDa protein, has a basic side isoform, pI 8.4. To examine whether the dephosphorylated CS has ability to form a 14 nm filament, the dephosphorylated CS was incubated under 14 nm filament-assembly conditions. CS was incubated with phosphatase for different time periods to induce pI 8.4 isoform by a partial dephosphorylation.

To remove phosphatase that disturbed the electron microscopic observation, phosphatase was removed with a

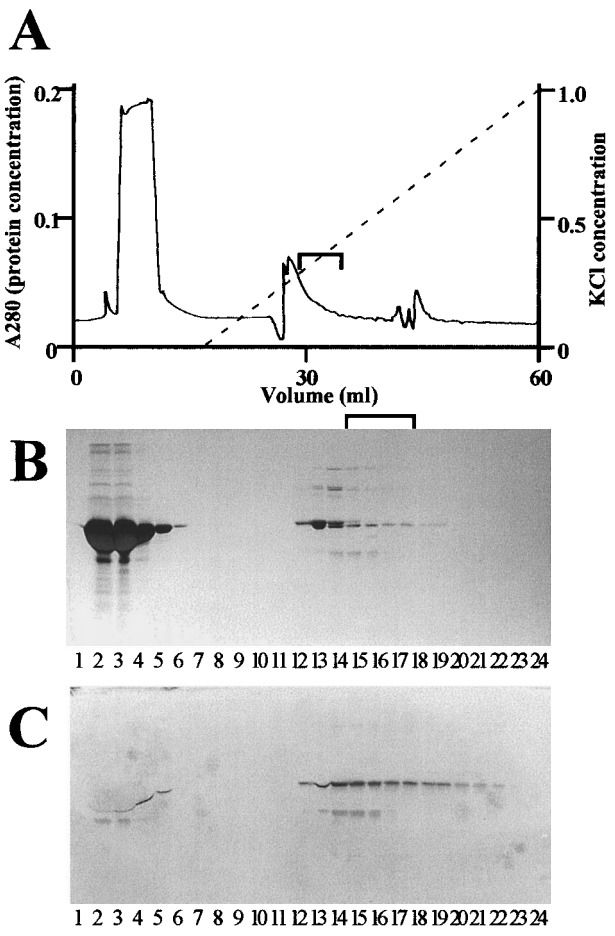

Fig. 3. Mono S column chromatography of the dephosphorylated citrate synthase. After dephosphorylation of CS for 4 hr, phosphatase was removed by Mono S column chromatography (A). A solid line and broken line represent protein concentration monitored at 280 nm (A 280) and KCl concentration, respectively. Peaks as indicated a bracket in "A" contained the dephosphorylated CS. Aliquots of the fractions were subjected to SDS-PAGE and silver stained (B). The fractions were also analyzed by immunoblotting with anti-14FP antibody (C). Lanes 1–24, Mono S column chromatographic fractions in (A). Lanes 15–18, CS fraction used the following experiment, indicating as brackets in (A) and (B).

Mono S ion exchange column chromatography. CS was dephosphorylated and loaded on to a Mono S column. The results of the elution pattern, SDS-PAGE, and immunoblotting were shown in Fig. 3. Dephosphorylated CS was recovered the peak from 250 to 350 mM KCl, and used for the following filament-assembly experiment. This peak contained single major band of CS as revealed by SDS-PAGE and reacted strongly to an anti-14FP antibody.

The partially dephosphorylated CS had an additional pI 8.4 isoform (Fig. 4B), while the native CS comprised the pI 8.0 and 7.7 isoforms (Fig. 4A). The completely dephosphorylated CS comprised the pI 8.8–9.0 isoform (Fig. 4C). The native 14FP and the native CS were taken to same filament assembly condition for a positive and negative control, respectively. The partially dephosphorylated CS and the completely dephosphorylated CS were incubated under 14-nm filament-assembly conditions for filament assembly assay. These were negative stained and observed by electron microscopy. The control 14FP formed bundles of 14-nm filaments (Fig. 5A) as previously reported (Takeda *et al.*, 1997). The native CS did not form filaments, but formed disordered aggregates and crystal-like structures (Fig. 5B, C). Interestingly, partial dephosphorylation of CS induced formation of many 14-nm filaments just as observed with the

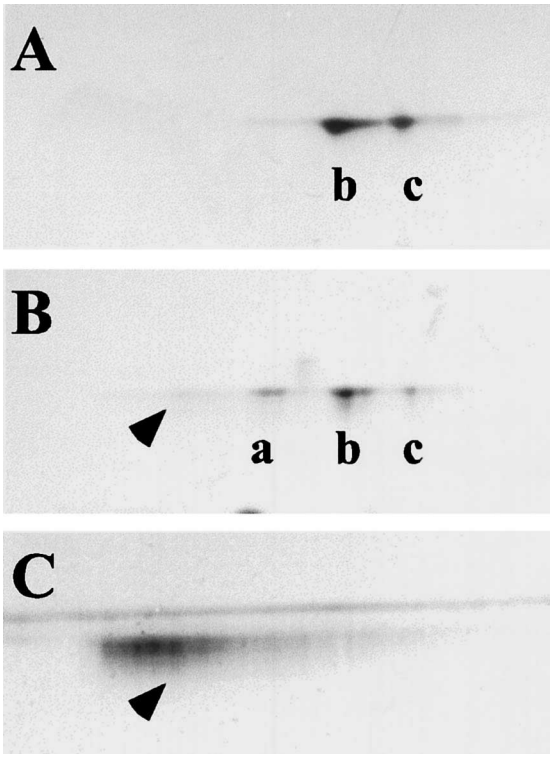

Fig. 4. Two-dimensional gel analysis of dephosphorylated CS. After removal of phosphatase by column chromatography, the non-treated CS (A), CS dephosphorylated for 4 hr (B), and CS dephosphorylated for 12 hr (C) were subjected to 2-DE and the gels were silver stained. The acid end of the gel is to the right. In (A) and (B), “a”, “b”, and “c” denote the pI 8.4, 8.0 and 7.7 isoforms, respectively. Arrowheads in (B) and (C) indicate the pI 8.9–9.0 isoform.

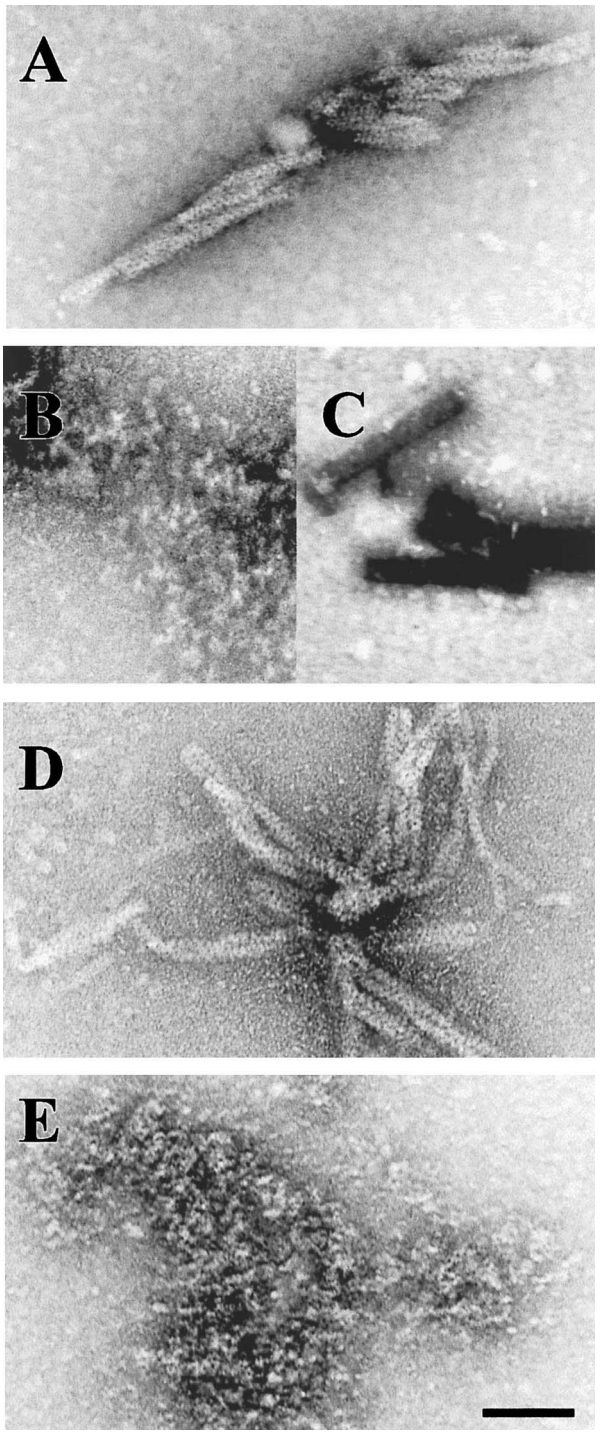

Fig. 5. Effect of dephosphorylation on assembly of CS. CS was treated with or without phosphatase for 4 or 12 hr, and incubated under 14-nm filament-assembly conditions after removal of phosphatase. These samples were observed by electron microscopy after negative staining. The purified 14FP formed 14-nm filaments (A). The non-treated CS formed aggregates and crystal like structures (B and C). The partially dephosphorylated CS formed 14-nm filaments (C). The completely dephosphorylated CS failed to form filaments (D). Bar represents 100 nm.

native 14FP (Fig. 5D). However the completely dephosphorylated CS failed to form filaments, but formed disordered aggregates similar to the native CS (Fig. 5E). These results indicate that the pI 7.7 and 8.0 isoforms are not sufficient to form the 14-nm filaments and that the acquisition of the pI 8.4 isoform is necessary for 14-nm filament assembly.

DISCUSSION

In this study, we demonstrated that dephosphorylation of isoforms reduced the citrate synthase activity of 49kDa protein. However by dephosphorylation, the enzyme form of 49kDa protein (CS) obtained the ability to form 14 nm filaments that was identical to cytoskeletal form of 49kDa protein (14FP). Besides 49kDa protein, there are no such reports that multifunctional protein sharing the ability of an enzyme and cytoskeleton.

14FP as a cytoskeleton has been reported to be involved in the formation of pronuclei, pronuclear exchange, and fertilization during conjugation (Numata *et al*., 1985; Takagi *et al*., 1991), while CS functions as a TCA cycle enzyme in mitochondria (Kojima *et al*., 1997). That is to say, the functions of 14FP and CS are involved in sexual reproduction and energy generation in mitochondria, respectively. Our results suggest that the unique regulations of these pivotal functions are due to phosphorylation and dephosphorylation. Since the 14FP purified as the filament comprise three isoforms and shares two isoforms with CS, it is likely that the third isoform is the key factor for the filament assembly. It is most likely to predict that the third isoform functions to nucleation of the filament or to stabilize and maintain the filament.

Phosphorylation and dephosphorylation regulate enzymatic activities of various enzymes, such as glycogen phosphorylase (Johnson and Barford, 1990; Barford *et al*., 1991), phosphorylase kinase (Johnson and Barford, 1990; Barford *et al*., 1991), and acetyl-CoA carboxylase (Thampy and Wakil, 1988). On the other hand, there are examples that assembly of filaments is as well as regulated by phosphorylation and dephosphorylation. Neurofilaments are major intermediate filaments expressed in neurons, and their assembly is regulated by phosphorylation and dephosphorylation (Gonda *et al*., 1990; Saito *et al*., 1995). Reorganization of vimentin and glial fibrillary acidic protein during mitosis are also regulated by phosphorylation (Inagaki *et al*., 1987; Matsuzawa *et al*., 1995). *Tetrahymena* 49kDa protein is the candidate for novel type of protein that simply regulates both enzymatic activity and filament formation by phosphorylation and/or dephosphorylation.

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