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F-Actin Bundling Activity of *Tetrahymena* Elongation Factor 1α Is Regulated by Ca^{2+}/Calmodulin[1]

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Translation elongation factor 1α (EF-1α) catalyzes the GTP-dependent binding of aminoacyl-tRNA to the ribosome. Previously, *Tetrahymena* 14-nm filament-associated protein was identified as EF-1α [Kurasawa *et al.* (1992) *Exp. Cell Res.* 203, 251-258]. This and several other studies suggest that EF-1α functions not only in translation but also in regulation of some part of the cytoskeleton. *Tetrahymena* EF-1α bound to F-actin and induced bundling of F-actin. We investigated the effects of GTP/GDP and Ca^{2+}/calmodulin on F-actin bundling activity of EF-1α. The presence of GTP, GDP, or guanylyl-imidodiphosphate (GMP-PNP) slightly decreased the amount of EF-1α which bound to F-actin, but each had virtually no effect on the F-actin bundling activity. The formation of F-actin bundles by EF-1α was Ca^{2+}-insensitive. In the absence of Ca^{2+}, calmodulin did not bind to EF-1α and F-actin. On the other hand, in the presence of Ca^{2+}, calmodulin directly bound to EF-1α but did not have any serious influence on EF-1α/F-actin binding. Under the conditions, electron microscopy demonstrated that Ca^{2+}/calmodulin completely inhibited the F-actin bundling by EF-1α. These results indicate that Ca^{2+}/calmodulin regulates the F-actin bundling activity of EF-1α without inhibition of the binding between EF-1α and F-actin.

Key words: calmodulin, cytoskeletal regulation, elongation factor 1α, F-actin bundling, *Tetrahymena*.

EF-1α catalyzes the GTP-dependent binding of aminoacyl-tRNA to the ribosome acceptor site in the peptide elongation phase of protein synthesis (*1, 2*). EF-1α is an abundant cellular protein with a molecular mass of about 50 kDa, a very basic isoelectric point (pI = 9.0), and is highly conserved among different species in eukaryotes (*3*).

Several studies suggest that EF-1α has the following novel functions related to microtubules. In sea urchins, EF-1α or EF-1α-like protein associates with microtubules and is closely correlated to the nucleation of astral microtubules (*4, 5*). In carrot, a homolog of EF-1α has microtubule bundling activity regulated by a Ca^{2+}/calmodulin-sensitive mechanism (*6*). In *Xenopus*, EF-1α has microtubule severing activity (*7*).

EF-1α also participates in the regulation of another cytoskeletal protein, actin. An F-actin bundling protein ABP-50 from *Dictyostelium* (*8*) has been identified as EF-1α from cDNA sequencing data and detection of the activity of polypeptide chain elongation (*9*). Following stimulation of cells with a chemoattractant, ABP-50/EF-1α becomes concentrated in filopodia, a type of pseudopod supported by an actin bundle (*10*). Another study indicated that, in *Physarum polycephalum*, 52-kDa protein induced co-bundling of F-actins and microtubules *in vitro*, and that the 52-kDa protein cross-reacted with antibody against yeast EF-1α (*11*).

In *Tetrahymena*, 14-nm filament protein has dual functions as a citrate synthase in mitochondria (*12-14*) and as a cytoskeletal protein involved in oral morphogenesis (*15, 16*) and in pronuclear behavior during conjugation (*17, 18*). The 14-nm filament protein was previously prepared from an extract of *Tetrahymena* acetone powder by polymerization and depolymerization (*19*). The 14-nm filament protein fraction contained two 49-kDa proteins whose isoelectric points were 8.0 and 9.0 (*20*). From the N-terminal amino acid sequences, the pI 8.0 protein was identified as previously reported 14-nm filament protein/citrate synthase, but the N-terminal sequence of the pI 9.0 protein had no similarity with that of the pI 8.0 protein (*20*). From the cloning and sequencing of the pI 9.0 protein gene from a *Tetrahymena pyriformis* cDNA library, we identified the pI 9.0 protein as EF-1α (*20*). *Tetrahymena* EF-1α is considered to be a 14-nm filament-associated protein since EF-1α co-purifies with 14-nm filament protein during two cycles of an assembly and disassembly purification protocol.

We have been interested in the function of EF-1α as a regulator of cytoskeletons and in the mechanism for the regulation of its multiple functions. *Tetrahymena* EF-1α

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Abbreviations: DTT, dithiothreitol; EF-1α, elongation factor 1α; GMP-PNP, guanylyl-imidodiphosphate; 2-ME, 2-mercaptoethanol; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloro-methyl ketone.

has been purified to investigate its possible function in cytoskeleton regulation (*21*). Recently the roles of EF-1α in 14-nm filament formation were studied, but EF-1α had no effect on the formation of 14-nm filament *in vitro* (*21*), and no other regulatory effect on the 14-nm filament has been found. Therefore, it is necessary to determine whether *Tetrahymena* EF-1α can interact with other cytoskeletal proteins, actin and tubulin.

In this study, we report that *Tetrahymena* EF-1α binds to F-actin and induces F-actin bundles *in vitro*. We further describe evidence for the regulation of F-actin bundling activity of EF-1α. Two possible regulators, GTP/GDP and Ca^{2+}/calmodulin, were tested. Here, we demonstrate that the F-actin bundling activity of EF-1α is clearly regulated by Ca^{2+}/calmodulin but not regulated by GTP/GDP.

MATERIALS AND METHODS

Cell Culture—Cultivation of *T. pyriformis* (strain W) was performed as described (*22*).

Gel Electrophoresis—SDS-PAGE was performed on a 10 or 15% running gel with a 3% stacking gel according to Laemmli (*23*). Native gel electrophoresis was performed on an alkaline glycerol gel (*24*) with two layers (5 and 10% gels) to detect both the faster migrating band corresponding to calmodulin and the slower migrating band corresponding to the EF-1α/calmodulin complex. Gels were stained with Coomassie Brilliant Blue R-250.

Preparation of Tetrahymena EF-1α, Tetrahymena Actin, and Tetrahymena Calmodulin—*Tetrahymena* EF-1α was purified by the method described before (*21*). *Tetrahymena* actin was purified according to the procedure of Hirono *et al.* (*25*). *Tetrahymena* calmodulin was bacterially expressed as a glutathione S-transferase (GST) fusion protein, and was purified by the method described before (*26*).

GTP-Binding Assay—GTP-binding assays were performed by the method described by Nagata *et al.* (*27*) with some modifications. The indicated amount of EF-1α, 0.15 nmol [α-^{32}P]GTP, and 7 nmol GTP were mixed in a reaction buffer [40 mM Tris-HCl, 10 mM $Mg(CH_3COO)_2$, 0.2 mM DTT, 0.5 M NH_4Cl, 1 mg/ml bovine serum albumin, 25% glycerol] in a final volume of 0.1 ml. After incubation at 37°C for 9 min, the sample was diluted with 1 ml of cold wash buffer [40 mM Tris-HCl, 5 mM $Mg(CH_3COO)_2$, and 0.1 M NH_4Cl, pH 7.5] and immediately filtered through a nitrocellulose membrane filter. The filter was washed twice with 5 ml of cold wash buffer and dried, and radioactivity retained on the filter was determined in a liquid scintillation counter. In a competition experiment, 40 μg EF-1α, 0.6 nmol [α-^{32}P]GTP, and 28 nmol GTP were added in the reaction buffer in a final volume of 0.8 ml. After incubation at 37°C for 5 min, which was long enough to saturate the binding of GTP, excess non-labeled ATP (500 nmol) or GTP (500 nmol) was added in the reaction mixture as a competitor. An aliquot (100 μl) of the reaction mixture was taken at indicated times and assayed by the method described above.

Protein Concentration—Protein concentration was determined by the method of Bradford (*28*) with bovine serum albumin as a standard.

Co-Sedimentation Experiment—EF-1α and calmodulin were dialyzed against MES buffer (10 mM MES, 0.75 mM 2-ME, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM KCl, 5% glycerol, 0.01 mM TLCK, 5 μg/ml leupeptin, 0.3 mM PMSF, pH 6.3). F-Actin was dialyzed against the MES buffer containing 100 mM KCl and 0.5 mM ATP. Three micromolar EF-1α, or 3 μM EF-1α preincubated with 4 μM calmodulin, was mixed with 3 μM *Tetrahymena* F-actin in the MES buffer and incubated at 26°C for 30 min, then ultracentrifuged at 250,000 $\times$ *g* for 30 min. The supernatant and pellet of the ultracentrifugation were analyzed by SDS-PAGE. The quantity of EF-1α and actin in individual fractions was determined with a scanning densitometer (Bio Image, Millipore Investment Holdings Limited).

Negative Staining Electron Microscopy—EF-1α, calmodulin, and F-actin were mixed and incubated as in the co-sedimentation experiment except for the incubation time (20 min). The mixture was mounted on a carbon-coated grid and negatively stained with 4% uranyl acetate. Grids were examined with a JEOL 100CXII electron microscope at an accelerating voltage of 80 kV.

RESULTS

Binding of GTP to Tetrahymena EF-1α—Purified *Tetrahymena* EF-1α used in this study is shown in Fig. 1A. To ascertain whether purified *Tetrahymena* EF-1α binds to GTP, GTP-binding capacity of *Tetrahymena* EF-1α was examined by the nitrocellulose filter method (*27*). With increasing EF-1α there was a linear increase of bound [α-^{32}P]GTP (Fig. 1B). The binding of GTP with EF-1α was completed within 3 min and the ratio of EF-1α to GTP was

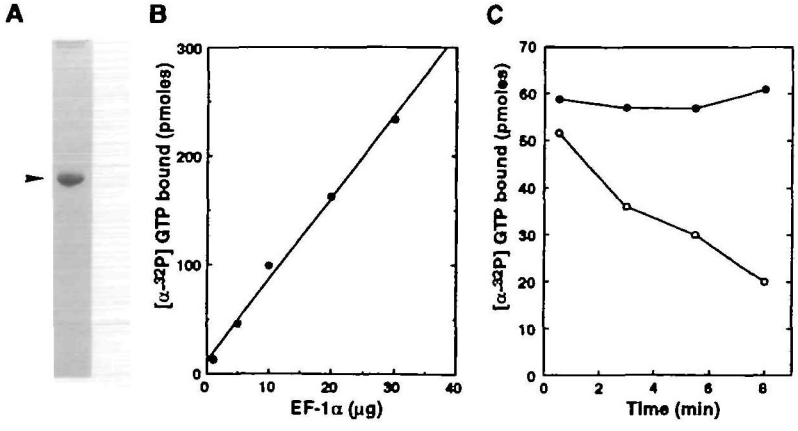

Fig. 1. **GTP-binding property of *Tetrahymena* EF-1α.** A: Purified EF-1α was analyzed by SDS-PAGE. B: [α-^{32}P]GTP binding to EF-1α was assayed by nitrocellulose filter method (*27*). The indicated amount of EF-1α, 0.15 nmol [α-^{32}P]GTP, and 7 nmol GTP were mixed and incubated for 9 min at 37°C, and immediately filtered through a nitrocellulose membrane filter. After washing, the radioactivity retained on the filter was determined. C: Specificity of nucleotide binding to EF-1α was assayed by a competition experiment. After saturation of the binding of [α-^{32}P]GTP, excess of non-labeled ATP (solid circles) or GTP (open circles) was added to the mixture. The mixtures were assayed at indicated times after the addition of competitors by the method described above.

approximately 1 : 0.6 (data not shown). Since bound GTP is rapidly exchangeable with external GTP (*27*), the specificity of the nucleotide binding to EF-1α was examined by a competition experiment using non-labeled GTP or ATP as a competitor. [α-^{32}P]GTP binding to EF-1α was rapidly exchanged with non-labeled GTP, but not exchanged with non-labeled ATP (Fig. 1C). This result indicates that the nucleotide-binding property of the purified EF-1α is specific for GTP.

F-Actin Bundling Activity of Tetrahymena EF-1α—The interaction between EF-1α and F-actin was first found in *Dictyostelium discoideum* (*29*). *Dictyostelium* ABP-50/EF-1α was originally isolated as an F-actin bundling protein (*8*) and then identified as EF-1α (*9*). To examine the interaction between purified *Tetrahymena* EF-1α and *Tetrahymena* F-actin, EF-1α and/or F-actin were mixed and incubated, and the mixture was subsequently ultracentrifuged. In the case of EF-1α alone, most of the EF-1α was not precipitated by this high speed centrifugation (Fig. 2A, lane 2). In the presence of F-actin, EF-1α was co-precipitated with F-actin (Fig. 2A, lane 4). The binding of *Tetrahymena* EF-1α to F-actin was saturated at a stoichiometry of about 1 EF-1α per 1 actin in the filament (data not shown).

To investigate the effect of *Tetrahymena* EF-1α on F-actin organization, the mixture of EF-1α and *Tetrahymena* F-actin was observed by electron microscopy after negative staining. EF-1α alone does not form any filamentous structure (*21*). The mixture of EF-1α and F-actin contained many F-actin bundles (Fig. 2B, panel b). These results demonstrate that *Tetrahymena* EF-1α binds to F-actin and induces bundling of F-actin as well as *Dictyostelium* ABP-50/EF-1α. F-actin bundles formed by *Tetrahymena* EF-1α looked very tight, the same as bundles formed by *Dictyostelium* EF-1α. In the mixture of EF-1α and F-actin, single actin filaments observed outside of bundles were straighter (Fig. 2B, panel c) than actin filaments in F-actin alone (Fig. 2B, panel a).

The Effect of GTP and GDP on the Regulation of F-Actin Bundling Activity of EF-1α—The activity of EF-1α as a peptide elongation factor is regulated by GTP/GDP (*1, 2*). The GTP-bound form of EF-1α is the active form for the peptide elongation. The bound GTP is hydrolyzed in the

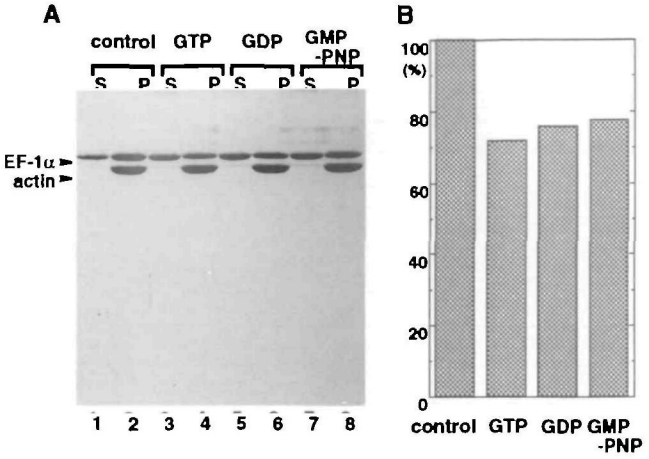

Fig. 3. **The effects of GTP, GDP, and GMP-PNP on F-actin binding of *Tetrahymena* EF-1α.** A: In the absence of guanine nucleotide (lanes 1 and 2) or in the presence of 1 mM GTP (lanes 3 and 4), 1 mM GDP (lanes 5 and 6), or 1 mM GMP-PNP (lanes 7 and 8), 3 μM EF-1α and 3 μM F-actin were incubated and binding of EF-1α to F-actin was analyzed by the co-sedimentation experiment as in Fig. 2. Supernatant and pellet for each condition are shown by S and P, respectively. Bands corresponding to EF-1α and actin are shown. B: The relative amount of EF-1α band in each pellet fraction shown in Fig. 3A was analyzed by densitometry. Histograms represent percentages of binding of EF-1α to F-actin in the presence of GTP, GDP, and GMP-PNP. In the absence of guanine nucleotide (control), the amount of EF-1α band in pellet fraction is taken as 100%, and those in other conditions are expressed as the relative ratios (percentages).

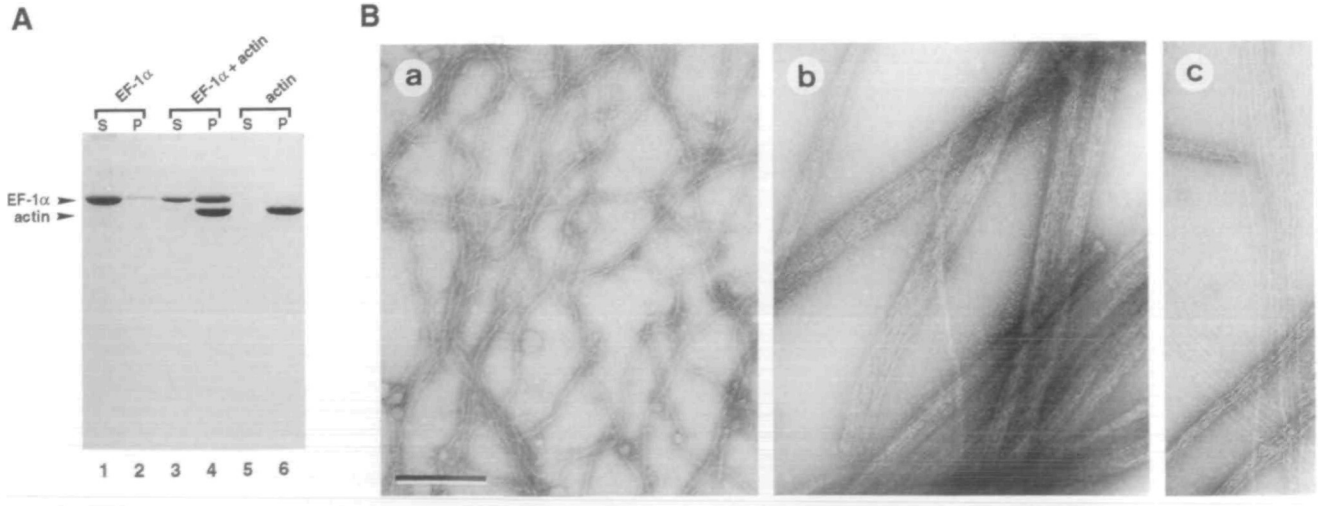

Fig. 2. **Binding of *Tetrahymena* EF-1α to F-actin and F-actin bundle formation by EF-1α.** A: 3 μM *Tetrahymena* EF-1α and 3 μM *Tetrahymena* F-actin were mixed and incubated at 26°C for 30 min, then ultracentrifuged at 250,000 × *g* for 30 min. The supernatant and pellet of the ultracentrifugation were analyzed by SDS-PAGE (15% gel). Supernatant (S) and pellet (P) of the sample containing EF-1α alone (lanes 1 and 2), EF-1α and F-actin (lanes 3 and 4), or F-actin alone (lanes 5 and 6) were indicated. Bands corresponding to EF-1α and actin are shown. B: 3 μM F-actin alone (panel a) or a mixture of 3 μM F-actin and 3 μM EF-1α (panels b and c) was incubated at 26°C for 20 min, then each mixture was analyzed by electron microscopy after negative staining. The bar represents 200 nm.

peptide elongation step, leading to the inactive GDP-bound form of EF-1α. Then GDP-bound form is exchanged to active GTP-bound form by co-factors of EF-1α.

Therefore, the effect of GTP, GDP, or GMP-PNP, a non-hydrolyzable analog of GTP, on the F-actin bundling activity of EF-1α was examined by co-sedimentation experiment. The result showed that presence of GTP, GDP, or GMP-PNP decreased the amount of EF-1α co-precipitated with F-actin to 71.9, 75.6, or 77.4% of the value without nucleotides, respectively (Fig. 3). However, electron microscopic observation showed that these nucleotides had no influence on F-actin bundle formation (data not shown). We consider that GTP and GDP are not essential for the regulation of F-actin bundling activity of *Tetrahymena* EF-1α.

Regulation of F-Actin Bundling Activity of EF-1α by Ca^{2+}/Calmodulin—Recently, Kaur and Ruben (*30*) have reported that EF-1α is a calmodulin-binding protein in *Trypanosoma*. In addition, microtubule bundling activity of carrot homolog of EF-1α was regulated by Ca^{2+}/calmodulin (*6*). Therefore, it is necessary to examine whether F-actin bundling activity of EF-1α is regulated by Ca^{2+}/calmodulin.

We firstly examined the interaction between *Tetrahymena* EF-1α and calmodulin. *Tetrahymena* EF-1α and *Tetrahymena* calmodulin were mixed and incubated in the presence of Ca^{2+} or EGTA, and subsequently analyzed by native gel electrophoresis using an alkaline glycerol gel. Ca^{2+}/calmodulin migrates slower than Ca^{2+}-free calmodulin in the native gel (*31*) (Fig. 4, lanes 1 and 7). EF-1α alone

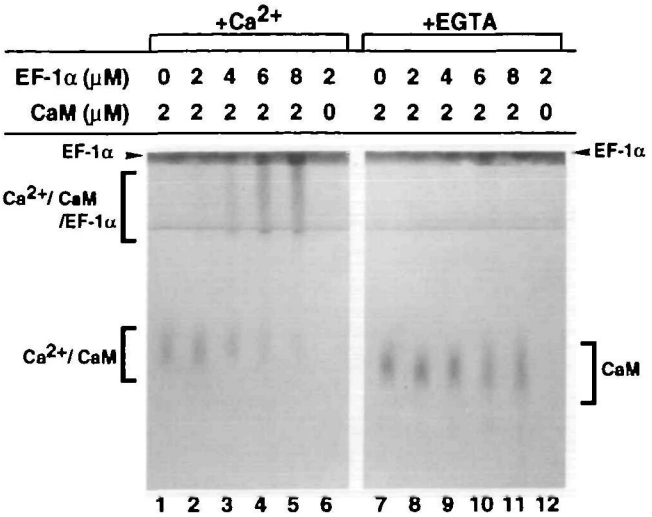

Fig 4 **The direct binding of Ca^{2+}/calmodulin to *Tetrahymena* EF-1α.** Indicated amounts of *Tetrahymena* EF-1α and/or *Tetrahymena* calmodulin were incubated at 0°C for 40 min in the MES buffer (see "MATERIALS AND METHODS") containing 1 mM $CaCl_2$ (lanes 1-6) or 1 mM EGTA (lanes 7-12). The mixture was subjected to native gel electrophoresis using an alkaline glycerol gel with two layers, a 5% gel (upper part of the plate) and a 10% gel (lower part of the plate) Bands corresponding to EF-1α, Ca^{2+}/calmodulin, Ca^{2+}-free calmodulin, and Ca^{2+}/calmodulin/EF-1α complex are indicated by EF-1α, Ca^{2+}/CaM, CaM, and Ca^{2+}/CaM/EF-1α, respectively.

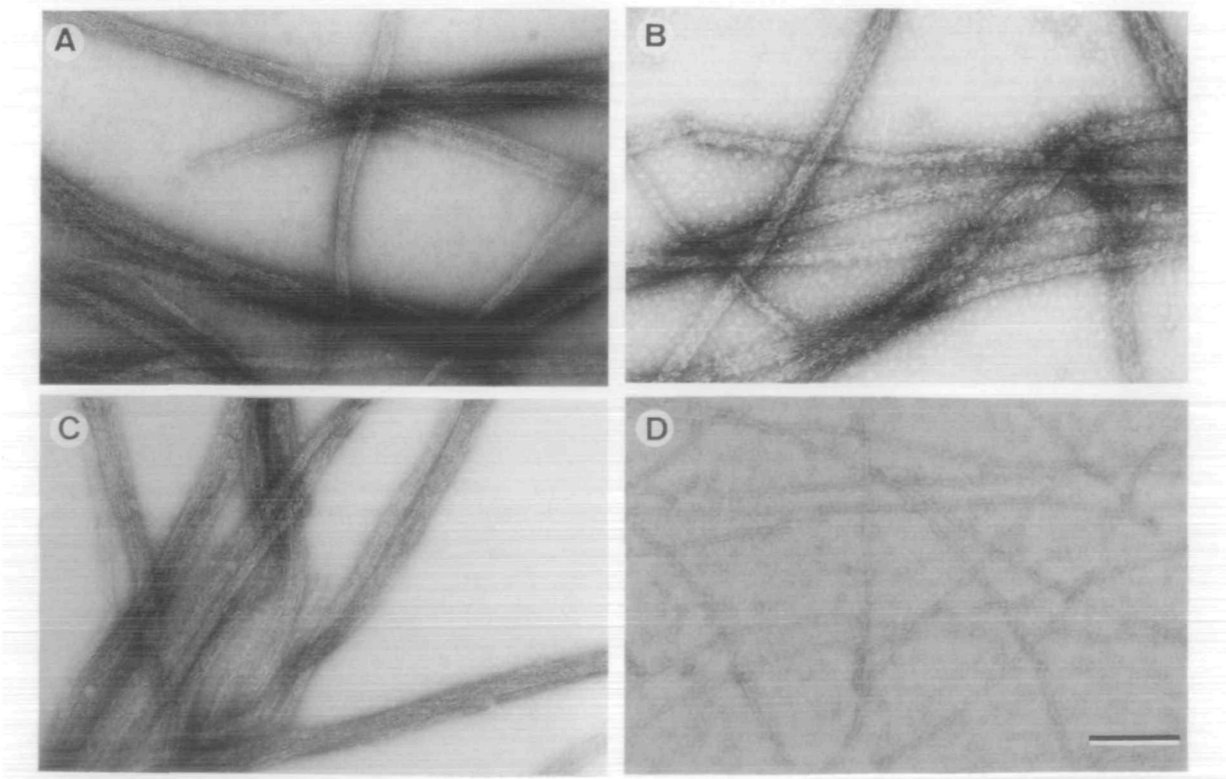

Fig. 5. **Regulation of the F-actin bundling activity of EF-1α by Ca^{2+}/calmodulin.** Three micromolar *Tetrahymena* EF-1α preincubated without (panels A and C) or with 4 μM calmodulin (panels B and D) were mixed with 3 μM *Tetrahymena* F-actin in the presence of 1 mM EGTA (panels A and B) or 1 mM $CaCl_2$ (panels C and D) Mixtures were incubated at 26°C for 20 min and analyzed by electron microscopy after negative staining. The bar represents 200 nm.

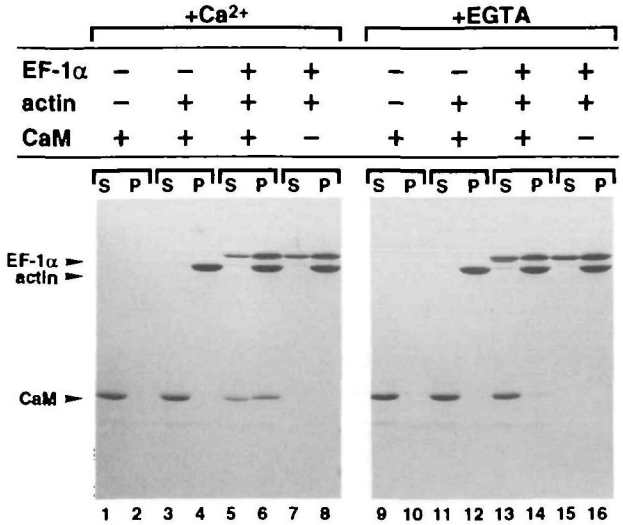

Fig 6 **Binding of Ca^{2+}/calmodulin to EF-1α/F-actin complex.** Four micromolar calmodulin alone (lanes 1, 2, 9, and 10), 3 μM F-actin and 4 μM calmodulin (lanes 3, 4, 11, and 12), 3 μM EF-1α preincubated with 4 μM calmodulin and 3 μM F-actin (lanes 5, 6, 13, and 14), and 3 μM EF-1α and 3 μM F-actin (lanes 7, 8, 15, and 16) were analyzed in the presence of 1 mM $CaCl_2$ (lanes 1-8) or 1 mM EGTA (lanes 9-16) by co-sedimentation experiment as in Fig 2. Supernatant and pellet for each condition are shown by S and P, respectively. Bands corresponding to EF-1α, actin, and calmodulin are indicated.

hardly migrated into the gel (Fig. 4, lanes 6 and 12), probably because of its high isoelectric point (*32*). When calmodulin was incubated with EF-1α under Ca^{2+} conditions, the protein in the band corresponding to Ca^{2+}/calmodulin decreased and another band appeared in the upper part of the gel (Fig. 4, lanes 2-5). This band was considered to be Ca^{2+}/calmodulin/EF-1α complex, because, as the concentration of EF-1α increased, protein migrating in this band increased while that of the band corresponding to Ca^{2+}/calmodulin decreased (Fig. 4, lanes 2-5). In addition, using SDS-PAGE, we observed that the gel slice of this band contained EF-1α and calmodulin (data not shown). No change was observed under EGTA conditions (Fig. 4, lanes 8-11). These results demonstrate the direct interaction between *Tetrahymena* EF-1α and Ca^{2+}/calmodulin.

Next, to investigate the effect of Ca^{2+}/calmodulin on F-actin bundling activity of EF-1α, *Tetrahymena* EF-1α was preincubated with *Tetrahymena* calmodulin under Ca^{2+} or EGTA conditions and then F-actin was added to the mixture. Each mixture was examined by electron microscopy and the co-sedimentation experiment.

Electron microscopy showed that, in the absence of calmodulin, EGTA or Ca^{2+} conditions had no effect on the F-actin bundling activity (Fig. 5, panels A and C), indicating that the bundling activity was Ca^{2+}-insensitive. On the contrary, in the presence of both Ca^{2+} and calmodulin, F-actin bundling activity of EF-1α was completely inhibited (Fig. 5, panel D), whereas calmodulin in the presence of EGTA had no effect on the bundling activity (Fig. 5, panel B). These results indicate that Ca^{2+}/calmodulin directly interacts with EF-1α and regulates the F-actin bundling activity of EF-1α.

Ca^{2+}-insensitivity of the formation of EF-1α/F-actin complex was further confirmed by co-sedimentation experiment (Fig. 6, lanes 7, 8, 15, and 16). In the presence of Ca^{2+}, calmodulin was co-precipitated with EF-1α/F-actin complex (Fig. 6, lanes 5 and 6), but not in the presence of EGTA (Fig. 6, lanes 13 and 14). The ternary complex formed in the presence of Ca^{2+} was composed of Ca^{2+}/calmodulin, EF-1α, and actin. Densitometric scan of the gel showed that the molar ratio of each component was 2.1 : 2.2 : 3. Since calmodulin with or without Ca^{2+} did not co-precipitate with F-actin alone (Fig. 6, lanes 3, 4, 11, and 12), Ca^{2+}/calmodulin possibly bound to EF-1α within the EF-1α/F-actin complex. As mentioned before, in the presence of EF-1α and Ca^{2+}/calmodulin, F-actin bundle formation by EF-1α is completely inhibited. However, it is worthy of note that the binding of EF-1α to F-actin is not affected by Ca^{2+}/calmodulin (Fig. 6, lane 6).

DISCUSSION

Previously, we identified *Tetrahymena* 14-nm filament-associated protein as EF-1α, based upon its cDNA sequencing data, which showed that the GTP-binding and tRNA-binding domains of EF-1α were well conserved (*20*). The purified *Tetrahymena* EF-1α was shown to have nucleotide-binding capacity specific for GTP (Fig. 1). This result confirms the identification of that protein as EF-1α.

The present experimental data demonstrated that purified *Tetrahymena* EF-1α was able to bind to *Tetrahymena* actin filaments and to induce actin filament bundles (Fig. 2). Dynamic bundle-formation of contractile ring actin filaments has been considered to be of utmost importance for the constriction of the division furrow in cytokinesis of *Tetrahymena* (*33, 34*), so that it is necessary to know the regulation mechanisms of F-actin bundling by EF-1α.

Concerning the regulation mechanisms, we first speculated that the regulation is effected by GTP-GDP exchange on EF-1α. In the translation system, EF-1α forms a complex with GTP and aminoacyl-tRNA before peptide elongation, and after peptide elongation, EF-1α forms a complex with only GDP hydrolyzed from GTP. Then, the GDP-bound form of EF-1α is changed to GTP-bound form by EF-1β/EF-1Γ complex, which is the GTP-GDP exchanging factor. As is well known, the GTP-bound form of EF-1α is active, whereas the GDP-bound form is inactive. Thus, the GTP-GDP exchange on EF-1α is an essential regulation system in translation. However, the present experimental results showed that GTP, GDP, and GMP-PNP exerted little or no influence upon F-actin binding and F-actin bundling of *Tetrahymena* EF-1α (Fig. 3). In addition, it was reported that the relationship between *Dictyostelium* ABP-50/EF-1α and F-actin was not affected by the presence of GTP or GDP (*10*). Thus, in spite of the fact that EF-1α is a GTP-binding protein, the function of EF-1α as a regulator of microfilaments appears to be independent of GTP.

Several recent reports have linked EF-1α with the microtubule cytoskeleton and/or the microfilament cytoskeleton (*35, 36*). In addition, a cytosolic calmodulin-binding protein from *Trypanosoma brucei* was identified as EF-1α (*30*). In carrot, an EF-1α homolog was shown to mediate the lateral association of microtubules by a Ca^{2+}/calmodulin-sensitive mechanism (*6*). Based on these findings, we secondly speculated that the functions of EF-1α family proteins as regulators in the organization of micro-

filaments as well as microtubules may be modulated by a Ca^{2+}/calmodulin system. To examine this hypothesis, we investigated interactions between EF-1α and F-actin and effects of Ca^{2+}/calmodulin on this interaction, by using purified *Tetrahymena* EF-1α, *Tetrahymena* actin, and *Tetrahymena* calmodulin. As a result, we found that F-actin bundling activity of EF-1α is clearly regulated by Ca^{2+}/calmodulin, as we had speculated (Fig. 5).

The essential role of Ca^{2+} in the microfilament system is well known. Ca^{2+}-sensitive actin binding proteins such as fragmin (*37*), severin (*38*), gelsolin (*39*), villin (*40*), and non-muscle α-actinin (*41*) regulate the microfilament system in non-muscle cells. In addition, Ca^{2+}/calmodulin regulates actin-activated myosin ATPase in smooth muscle cells and non-muscle cells *via* activation of myosin light chain kinase (*42*). In the present study, although F-actin binding and F-actin bundling activities of *Tetrahymena* EF-1α were Ca^{2+}-insensitive in the absence of calmodulin (Fig. 5), F-actin bundling activity was found to acquire Ca^{2+}-sensitivity in the presence of calmodulin (Fig. 5). As an experimental prerequisite for understanding the Ca^{2+}/calmodulin regulation of F-actin bundling, we demonstrated that Ca^{2+}/calmodulin bound directly to *Tetrahymena* EF-1α to form a complex (Fig. 4), but did not bind to *Tetrahymena* F-actin at all (Fig. 6). In other words, *Tetrahymena* EF-1α is a Ca^{2+}-dependent calmodulin-binding protein like *Trypanosoma* EF-1α. As a consequence of this finding, three possible models concerning the regulation of F-actin bundling are conceivable as schematically illustrated in Fig. 7. Model 1 is a so-called flip-flop model of Ca^{2+}/calmodulin regulation (*43-45*), which explains the activation and inactivation of the many kinds of Ca^{2+}/calmodulin-dependent enzymes known so far. However, this simple model does not seem to hold true for Ca^{2+}/calmodulin regulation of F-actin bundling activity of EF-1α, because in this model only F-actin should be recovered from the pellets in the co-sedimentation experiment in the presence of Ca^{2+}, calmodulin, and EF-1α, but in fact, calmodulin and EF-1α co-precipitated with F-actin (Fig. 6, lane 6). Ca^{2+}/calmodulin really bound to EF-1α within the F-actin/EF-1α complex and had no effect on binding of EF-1α to F-actin (Fig. 6, lane 6). To explain both this result and evidence that Ca^{2+}/calmodulin completely inhibits F-actin bundling activity of EF-1α (Fig. 5), the more complicated models 2 and 3 (Fig. 7) are proposed. In model 2, we speculate that EF-1α has two independent actin-binding domains within a single molecule and one of them is inactivated by Ca^{2+}/calmodulin binding. Edmonds *et al.* suggest that actin filament cross-linking and filament binding by EF-1α are separate events (*46*). On the other hand, in model 3, we speculate that EF-1α has only one actin-binding domain in a single molecule, but anti-parallel dimer formation is responsible for cross-linking the actin filaments, and that Ca^{2+}/calmodulin inhibits such a dimer formation.

To cross-link actin filaments, actin-binding proteins such as villin (*47*) and fimbrin (*48, 49*) contain two separate actin-binding domains in a single molecule. On the other hand, α-actinin has only one actin-binding domain in the N-terminal region (*50*). By the formation of an anti-parallel dimer, α-actinin acquires two actin-binding domains at its ends (*49, 51*). As far as EF-1α is concerned, it has one region which is homologous to the actin-binding domain of depactin (*9*), and therefore it might also form an anti-parallel dimer to get two actin-binding sites. This possibility might be supported by our result that the molar ratio of *Tetrahymena* EF-1α to actin in F-actin bundles was approximately 1 : 1. At present, model 2 cannot be excluded, but model 3 offers a better explanation of the present experimental results. To validate the possibility that F-actin bundling is brought about by EF-1α dimers and that Ca^{2+}/calmodulin inhibits dimer formation of EF-1α, we are now examining the relationships between EF-1α dimer formation and F-actin bundling and the effect of Ca^{2+}/calmodulin on the EF-1α dimer formation.

Finally, we stress the notion that EF-1α is an essential protein and is highly conserved during evolution. *Tetrahy-*

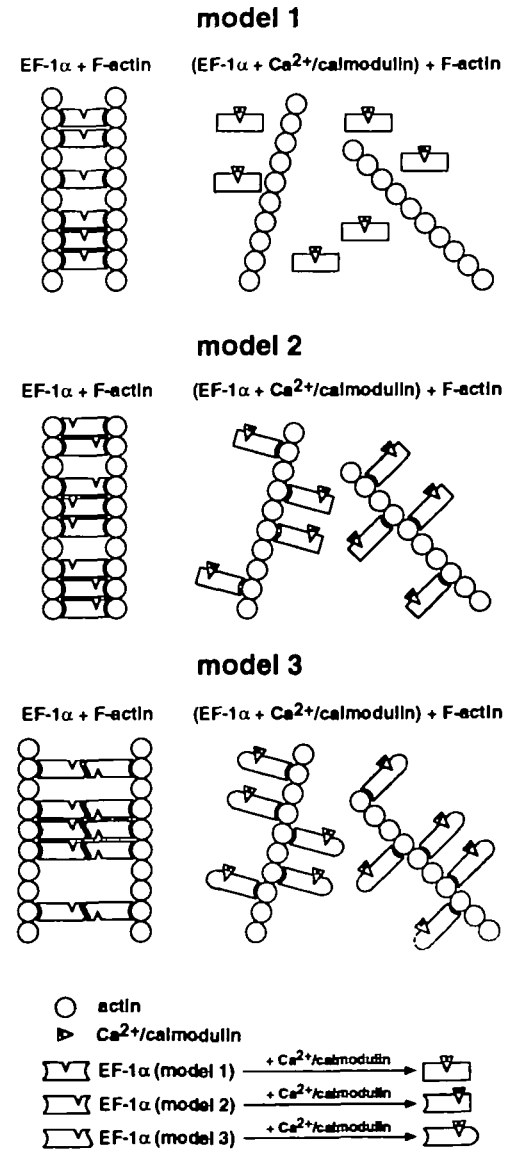

Fig. 7. **A schematic illustration of possible models for the regulation of F-actin bundling activity of EF-1α by Ca^{2+}/calmodulin.** model 1, a so-called flip-flop model (*45-47*); model 2, EF-1α monomer model; model 3, EF-1α dimer model. Left sides indicate the mixture of EF-1α and F-actin. Right sides indicate that F-actin was added to preincubated mixture of EF-1α and Ca^{2+}/calmodulin. For detailed explanation, see text.

mena EF-1α and *Dictyostelium* EF-1α have F-actin bundling activity, so that EF-1α must have acquired the F-actin bundling activity in the first stage of molecular evolution and maintained such activity to the present time. During evolution, *Tetrahymena* EF-1α has maintained a second role without gene duplication. Thus, we conclude that *Tetrahymena* EF-1α is a conservative multifunctional protein.

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