

# Roles of Three Domains of *Tetrahymena* eEF1A in Bundling F-actin

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The conventional role of eukaryotic elongation factor 1A (eEF1A) is to transport aminoacyl tRNA to the A site of ribosomes during the peptide elongation phase of protein synthesis. eEF1A also is involved in regulating the dynamics of microtubules and actin filaments in cytoplasm. In *Tetrahymena*, eEF1A forms homodimers and bundles F-actin. Ca<sup>2+</sup>/calmodulin (CaM) causes reversion of the eEF1A dimer to the monomer, which loosens F-actin bundling, and then Ca<sup>2+</sup>/CaM/eEF1A monomer complexes dissociate from F-actin. eEF1A consists of three domains in all eukaryotic species, but the individual roles of the *Tetrahymena* eEF1A domains in bundling F-actin are unknown. In this study, we investigated the interaction of each domain with F-actin, recombinant *Tetrahymena* CaM, and eEF1A itself in vitro, using three glutathione-S-transferase-domain fusion proteins (GST-dm1, -2, and -3). We found that only GST-dm3 bound to F-actin and influences dimer formation, but that all three domains bound to *Tetrahymena* CaM in a Ca<sup>2+</sup>-dependent manner. The critical Ca<sup>2+</sup> concentration for binding among three domains of eEF1A and CaM were ≤100 nM for domain 1, 100 nM to 1 μM for domain 3, and >1 μM for domain 2, whereas stimulation of and subsequent Ca<sup>2+</sup> influx through Ca<sup>2+</sup> channels raise the cellular Ca<sup>2+</sup> concentration from the basal level of ~100 nM to ~10 μM, suggesting that domain 3 has a pivotal role in Ca<sup>2+</sup>/CaM regulation of eEF1A.

**Key words:** *Tetrahymena*, eukaryotic translation elongation factor 1A (eEF1A), actin, cytoskeleton, Ca<sup>2+</sup>, calmodulin

## INTRODUCTION

Eukaryotic elongation factor 1A (eEF1A; formerly EF-1 $\alpha$ ; EF1A in prokaryotes) is an abundant cytoplasmic protein that is conserved in all eukaryotes. It delivers aminoacyl-tRNA (aa-tRNA) to the A site of ribosomes during the peptide elongation phase of protein synthesis. Binding of eEF1A with the eEF1B guanine nucleotide exchange factor (GEF) complex leads to replacement of GDP with GTP, and on binding GTP, eEF1A can interact with aa-tRNA. Subsequently, the binding of eEF1A/GTP/aa-tRNA ternary complex with the ribosome triggers the GTPase activity of eEF1A and subsequent dissociation of eEF1A/GDP from the ribosome. Structural data show that EF1A and eEF1A consist of three domains (Andersen *et al.*, 2000, 2003), which are well conserved among species. Domain 1 is composed of about 200 amino acid residues and has a central  $\beta$  sheet surrounded by eight  $\alpha$  helices. The active site of the GTPase activity of eEF1A, the G domain, is in domain 1. Domains 2 and 3 each contain about 100 amino acid residues and have  $\beta$  barrels.

In addition to its role in translation elongation, eEF1A is involved in the regulation of microtubules and the actin cytoskeleton. In carrot, a homolog of eEF1A has microtu-

bule-bundling activity that is regulated by a Ca<sup>2+</sup>/calmodulin (CaM)-sensitive mechanism (Durso and Cry, 1994). The multifunctional eEF1A protein also participates in F-actin bundling. The actin-binding and F-actin-bundling activities of eEF1A were demonstrated initially in *Dictyostelium* (Yang *et al.*, 1990), and these functions of eEF1A are retained in other species, such as *Physarum polycephalum* (Itano and Hatano, 1991), *Tetrahymena* (Kurasawa *et al.*, 1996a), and fission yeast (Suda *et al.*, 1999). Because eEF1A binds to F-actin with relatively high affinity, eEF1A may be a potent regulator of the dynamics of the actin cytoskeleton. In *Dictyostelium*, binding between eEF1A and actin is sensitive to cellular parameters such as nucleotide concentration and pH (Edmonds *et al.*, 1995, 1998; Liu *et al.*, 1996). In vitro analysis of *Dictyostelium* eEF1A showed that domains 1 and 3 of eEF1A bind to F-actin, suggesting that eEF1A monomer can bundle F-actin (Liu *et al.*, 2002). In addition, *Dictyostelium* eEF1A has a role in anchoring  $\beta$ -actin mRNA to the actin cytoskeleton (Liu *et al.*, 2002).

*Tetrahymena* eEF1A bundles rabbit skeletal muscle F-actin in addition to *Tetrahymena* F-actin (Kurasawa *et al.*, 1996b). Using *Tetrahymena* eEF1A, *Tetrahymena* actin, and recombinant *Tetrahymena* CaM, Kurasawa *et al.* (1996a) demonstrated that Ca<sup>2+</sup>/CaM bound to eEF1A and completely inhibited subsequent formation of F-actin bundles. Furthermore, Bunai *et al.* (2006) demonstrated that *Tetrahymena* eEF1A dimerized and bundled F-actin but eEF1A monomer did not. Ca<sup>2+</sup>/CaM separated eEF1A dimers into monomers, thereby decreasing the affinity of

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eEF1A for F-actin;  $\text{Ca}^{2+}/\text{CaM}/\text{eEF1A}$  monomer complexes dissociated from F-actin. Conversely, chelation of  $\text{Ca}^{2+}$  by eEF1A monomer led to dimer formation (Bunai *et al.*, 2006). Therefore, the F-actin bundling activity of eEF1A is reversibly regulated by  $\text{Ca}^{2+}/\text{CaM}$ .

In *Tetrahymena*, the roles of the three eEF1A domains in F-actin bundling are unknown. To understand the roles of these domains in dimer formation and the interaction of *Tetrahymena* eEF1A with F-actin and  $\text{Ca}^{2+}/\text{CaM}$ , we prepared GST fusion proteins with domains 1, 2, and 3 in *Escherichia coli*. We then investigated the interaction of these three GST-domain fusion proteins (GST-dm1, -2, and -3) with F-actin, recombinant *Tetrahymena* CaM, and eEF1A in vitro. Domain 3 is responsible for forming dimers and binding F-actin, and all three domains bind CaM.

## MATERIALS AND METHODS

### Cell culture

*Tetrahymena pyriformis* (strain W) was cultivated in PYD medium (1% proteose peptone, 0.5% yeast extract, 0.87% D-glucose) at 26°C (Watanabe *et al.*, 1994).

### Preparation of *Tetrahymena* eEF1A and rabbit skeletal muscle actin

*Tetrahymena* eEF1A was purified as described previously (Takeda *et al.*, 1995). Rabbit skeletal muscle actin was purified according to the procedure of Pardee and Spudich (1982).

### Cloning of *Tetrahymena* eEF1A cDNA and site-directed mutagenesis

Full-length *Tetrahymena* eEF1A cDNA was obtained by polymerase chain reaction (PCR) amplification of an appropriate clone from a *Tetrahymena pyriformis* cDNA library (Edamatsu *et al.*, 1991). Primers for amplification (forward [containing a *Bam*HI site], 5'-CGCGGATCCATGGCTAGAGGT-3'; reverse, 5'-TCATTTGTCCTTCTTTTCGACTTTC-3') were designed based on the *Tetrahymena* eEF1A cDNA sequence (Kurasawa *et al.*, 1992). In *Tetrahymena* cells, TAA and TAG stop codons are used as glutamine codons (Horowitz and Gorovsky, 1985). There is only one TAG codon in *Tetrahymena* eEF1A cDNA, which was mutated to CAG by use of a mutated antisense primer, 5'-GCTTCGAATTCACCCTGGGGGGAAGC-3' (the mutated nucleotide is underlined). We used an *Eco*RI site near the mutation site to ligate the mutated fragment to the rest of the eEF1A sequence to reconstitute a full-length eEF1A cDNA. The ligated DNA fragment was cloned into the *Bam*HI and *Sma*I sites of pBluescript II KS. The orientation and sequence of the eEF1A insert was confirmed by the dideoxy chain termination method with an ABI Prism DNA sequencer 377 (Applied Biosystems, California, USA).

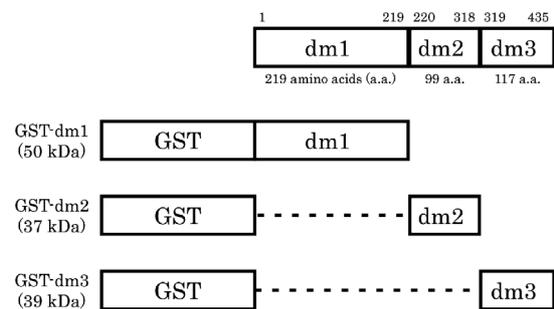
### Preparation of three GST-domain fusion proteins

After digestion of the pBlue-*Tetrahymena* eEF1A construct with *Bam*HI and *Xho*I, the DNA fragment was subcloned into the *Bam*HI and *Xho*I sites of pGEX-6P-1. By comparison with the sequence for *Dictyostelium* eEF1A (Yang *et al.*, 1990), we determined the three domains of *Tetrahymena* eEF1A (Fig. 1A). Insert DNAs for constructing the three GST-domain fusion proteins were prepared by

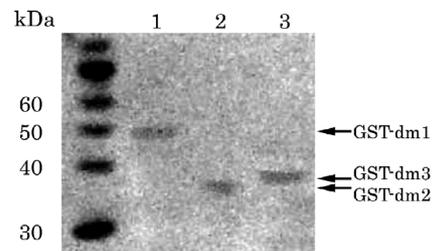
(A)



(B)



(C)



**Fig. 1.** (A) Amino-acid sequence of *Tetrahymena* eEF1A. The putative GTP-binding sites and the tRNA-binding site are underlined. The boundaries between the domains (dm) are indicated. The putative GTP-binding sites correspond to the consensus sequences GXXXXGK (a), DXXG (b), and NKXD (c), respectively. The putative tRNA-binding site corresponds to that of *E. coli* EF1A. (B) Schematic diagrams of the structures of GST-dm1, -2, and -3. The three domains of *Tetrahymena* eEF1A (domains 1, 2, and 3) are shown with their amino-acid residue numbers in parentheses. The calculated molecular masses of GST-dm1, -2, and -3 are 50, 37, and 39 kDa, respectively. (C) Purification of GST-dm1, -2, and -3. Proteins were resolved by SDS-PAGE. Molecular mass markers are shown on the left. (1) GST-dm1. (2) GST-dm2. (3) GST-dm3.

PCR amplification using pGEX-*Tetrahymena* eEF1A as the template. Forward primers (each containing a *Bgl*III site) for domain 1 (encoding amino acids 1 through 219), domain 2 (encoding amino acids 220 through 318), and domain 3 (encoding amino acids 319 through 435) were 5'-GGAAGATCTGGGATCCCGATGGATAGAGGT-3', 5'-GGAAGATCTGGGATCCCGGTCTCGATGCT-3' and 5'-GGAAGATCTGGGATCCCGAAGAACGACCCC-3', respectively. Reverse primers (each containing an *Xho*I site) for domains 1 and 2 were 5'-CCGCTCGAGCTATTCGACGAGGATGGGTCC-3' and 5'-CCGCTCGAGCTAGGCATCGGAAGCGACATT-3', respectively; the reverse primer for domain 3 was the pGEX 3' Sequencing Primer (Amersham Biosciences, Uppsala, Sweden). After digestion with *Bgl*III and *Xho*I, each DNA fragment was cloned into the *Bam*HI and *Xho*I sites of pGEX-4T-1. The orientation and sequence of all constructs were verified by automated sequencing using the dideoxy chain termination method.

The three GST-domain fusion proteins were expressed in *Escherichia coli* strain BL21. *Escherichia coli* cells harboring each of the constructs were grown in LB medium with 50 µg/ml ampicillin at 37°C, and the expression of fusion proteins was induced by adding 0.15 mM isopropyl-β-D-thiogalactoside for 4 to 6 h at 25°C. At the end of induction, the cells were harvested, and the cell pellet was washed once with washing buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.5) and then resuspended in lysis buffer (100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 10 mM β-mercaptoethanol, 10 mg/ml leupeptin, 10 mM p-toluenesulfonyl-L-lysine chloromethyl ketone, 1 mg/ml pepstatin A, 100 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 8.0). After sonication and centrifugation, the supernatant was incubated with Glutathione Sepharose 4B

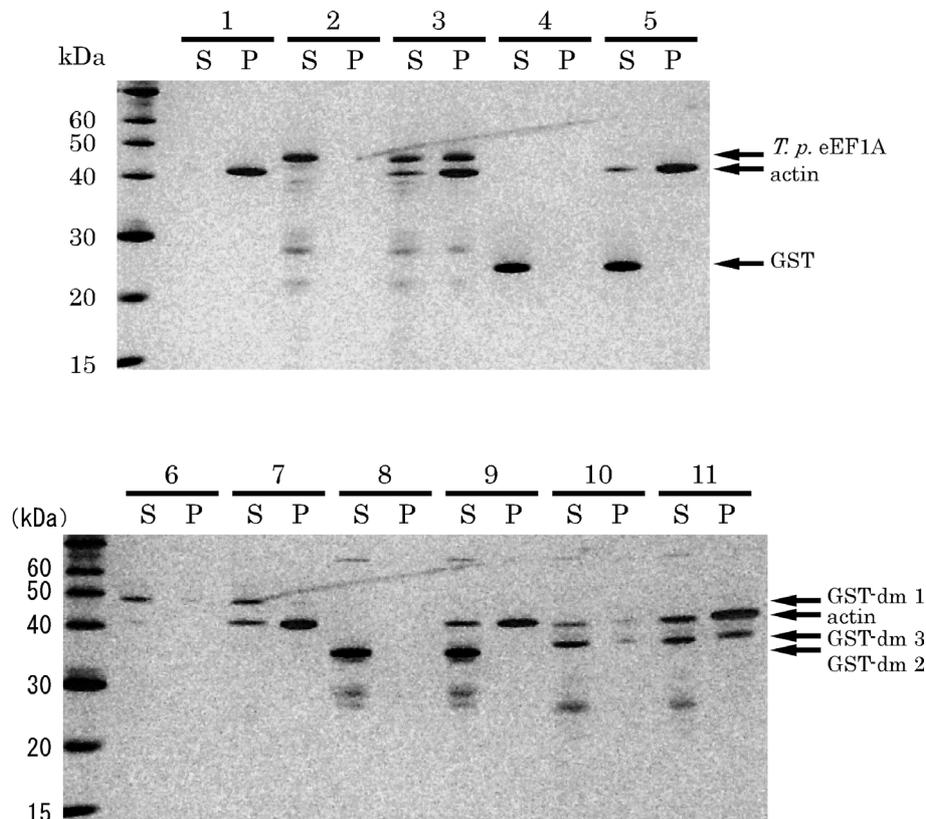
(Amersham Biosciences, Uppsala, Sweden) at 4°C for overnight. The beads were washed with washing buffer B (100 mM NaCl, 20 mM Tris-HCl, pH 8.0), and bound GST fusion proteins were eluted with elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0). The purified GST-domain proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### Preparation of recombinant *Tetrahymena* CaM

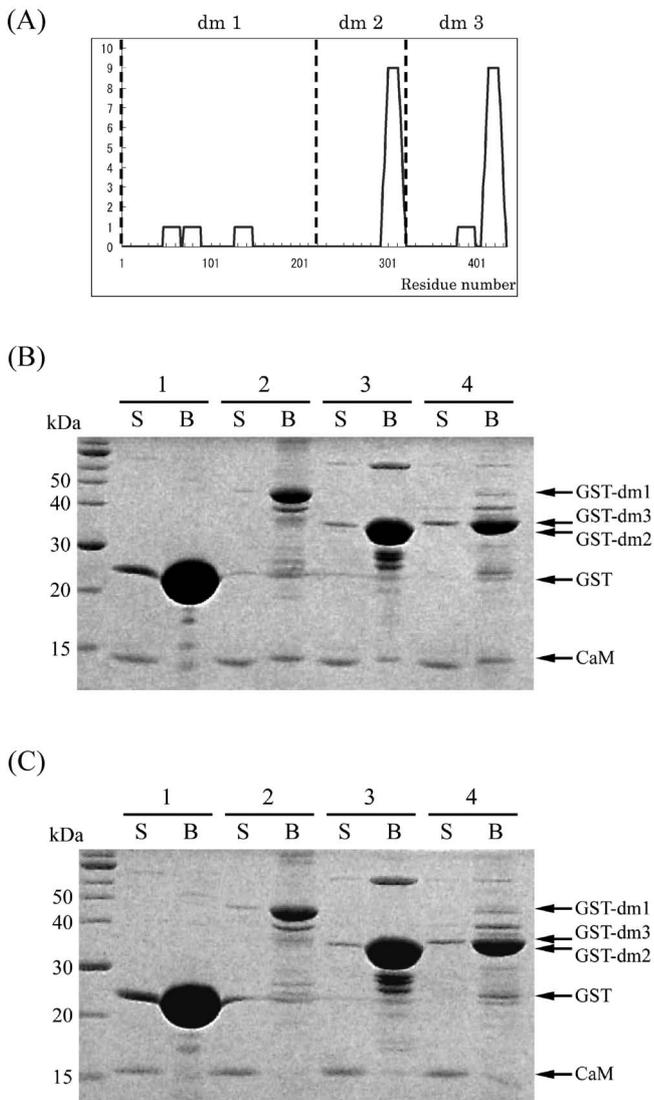
Recombinant *Tetrahymena* CaM was expressed as a GST fusion protein in *E. coli* and purified as described (Hanyu *et al.*, 1995). For GST pull-down assays, GST-*Tetrahymena* CaM fusion protein was cleaved with thrombin (Amersham Biosciences, Uppsala, Sweden) in thrombin buffer (150 mM NaCl, 2 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 8.0) at 4°C. Thrombin then was removed by incubation with Benzamidine Sepharose 6B (Amersham Biosciences, Uppsala, Sweden) at 4°C, and then recombinant *Tetrahymena* CaM was dialyzed in MES buffer (0.75 mM 2-mercaptoethanol, 2 mM MgCl<sub>2</sub>, 40 mM KCl, 5% glycerol, 10 mM 2-[N-morpholino] ethanesulfonic acid, pH 7.0).

#### Sequence analysis for putative CaM-binding sites of *Tetrahymena* eEF1A

Putative CaM-binding sites of *Tetrahymena* eEF1A were identified by sequence comparison with the Calmodulin Target Database (<http://calcium.uhnres.utoronto.ca/cgi-bin/ctdb/ctdb/home/html>). Normalized scores (0 to 9) are shown in Fig. 3a. A consecutive string of high values of normalized scores indicates the location of a putative CaM-binding site.



**Fig. 2.** Binding of GST-dm1, -2, and -3 to rabbit skeletal muscle F-actin. The supernatants (S) and pellets (P) from the actin cosedimentation assays were analyzed by SDS-PAGE. Bands corresponding to actin, *Tetrahymena* eEF1A, GST, and GST-dm1, -2, and -3 are stained with silver and shown by arrows. Molecular mass markers are shown on the left. (1) F-actin alone. (2) *Tetrahymena* eEF1A alone. (3) F-actin and *Tetrahymena* eEF1A. (4) GST alone. (5) F-actin and GST. (6) GST-dm1 alone. (7) F-actin and GST-dm1. (8) GST-dm2 alone. (9) F-actin and GST-dm2. (10) GST-dm3 alone. (11) F-actin and GST-dm3. Smaller bands in GST-dm2 and GST-dm3 are probably degradation products.



**Fig. 3.** The putative CaM-binding sites of *Tetrahymena* eEF1A and binding of GST-dm1, -2, and -3 to recombinant *Tetrahymena* CaM with or without Ca<sup>2+</sup>. **(A)** Sequence analysis for the putative CaM-binding sites of *Tetrahymena* eEF1A by the Calmodulin Target Database (<http://calcium.uhnres.utoronto.ca/cgi-bin/ctdb/ctdb/home/html>). Vertical axis shows normalized scores (0 to 9) and a consecutive string of high values indicates the location of a putative CaM-binding site. Horizontal axis shows amino acid residue numbers of *Tetrahymena* eEF1A. The boundaries between the domains are shown by the dotted lines. **(B)** and **(C)** GST pull-down assays with GST-dm1-3-bound beads and recombinant *Tetrahymena* CaM. Assays were performed in MES buffer containing 1 mM CaCl<sub>2</sub> (B) or 1 mM EGTA (C). The supernatants (S) and pellets of beads (P) from GST pull-down assays were analyzed by SDS-PAGE. Bands corresponding to GST-dm1, GST-dm2, GST-dm3, GST, and CaM are shown by arrows. The molecular mass markers are shown on the left. (1) GST-bound beads and CaM. (2) GST-dm1-bound beads and CaM. (3) GST-dm2-bound beads and CaM. (4) GST-dm3-bound beads and CaM.

#### High-speed and low-speed actin cosedimentation assays

To determine the actin-binding domain(s) of eEF1A, each GST-domain protein (2  $\mu$ M) in MES buffer was centrifuged at 100,000 $\times$ g for 30 min and the supernatant was mixed with rabbit skeletal muscle F-actin (2  $\mu$ M) in MES buffer. The mixtures were incubated for

30 min at 26°C and then centrifuged at high speed (100,000 $\times$ g for 30 min). The supernatants and pellets were analyzed by SDS-PAGE.

Because Ca<sup>2+</sup>/CaM inhibits eEF1A-associated F-actin bundle formation, we examined the effect of Ca<sup>2+</sup> concentration on the inhibition of F-actin bundle formation. We incubated 3  $\mu$ M F-actin, 3  $\mu$ M eEF1A, and 3  $\mu$ M CaM with various concentrations of Ca<sup>2+</sup> for 30 min at 26°C, and then centrifuged the reactions at low speed (12,000 $\times$ g for 30 min). The supernatants and pellets were analyzed by SDS-PAGE, and the amounts of actin in the supernatants and pellets were determined according to the strength of Coomassie Brilliant Blue R-250 staining of the protein bands.

#### GST pull-down assay

To identify the CaM-binding domain(s) of eEF1A, recombinant *Tetrahymena* CaM (2  $\mu$ M) was incubated with Glutathione Sepharose 4B beads bound to GST or GST-domain fusion proteins in MES buffer containing either 1 mM, 1  $\mu$ M, or 100 nM CaCl<sub>2</sub>, or 1 mM EGTA for 30 min at 26°C. After centrifugation at 500 $\times$ g for 3 min, the supernatants were removed and the beads were washed three times with MES buffer. The supernatants and the beads were analyzed by SDS-PAGE. To identify the domain(s) involved in dimer formation of eEF1A, *Tetrahymena* eEF1A (2  $\mu$ M) was incubated with Glutathione Sepharose 4B beads bound to GST or GST-domain fusion proteins in MES buffer for 30 min at 26°C and analyzed as described above.

#### Electrophoresis

SDS-PAGE was performed on 10% or 15% running gels with 3% stacking gels according to the method of Laemmli (1970). Gels were stained with silver or Coomassie Brilliant Blue R-250.

#### Protein concentration

Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976).

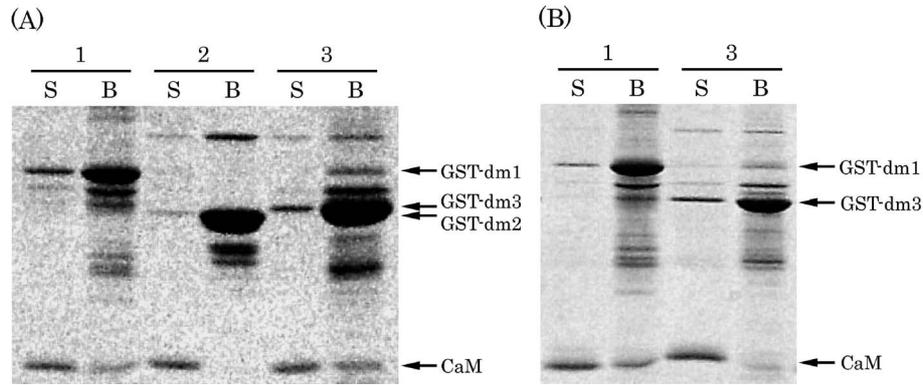
## RESULTS

#### Expression and purification of GST fusion proteins

GST-eEF1A fusion protein was expressed poorly in *E. coli* cells, mostly in the insoluble fraction of total bacterial lysate (data not shown). All three GST-domain constructs were expressed better and were more soluble than the GST-eEF1A fusion protein. However, GST-dm1, -2, and -3 all bound nonspecifically to Glutathione Sepharose 4B beads and were difficult to elute from them. To elute the fusion proteins from the beads, we minimized the volume of beads and prolonged the elution time; with these modifications, we were able to obtain satisfactory amounts of GST-dm1, -2, and -3. The GST-domain fusion proteins were purified almost to homogeneity (Fig. 1C).

#### F-actin-binding domain in *Tetrahymena* eEF1A

To identify the F-actin-binding domain of eEF1A, we performed cosedimentation assays with GST-dm1, -2, and -3; *Tetrahymena* eEF1A; and rabbit skeletal muscle F-actin (Fig. 2). In the absence of eEF1A or any of the fusion proteins, most of the F-actin was precipitated by centrifugation, whereas eEF1A, GST, or GST-domain fusion protein alone remained in solution. In the positive control, eEF1A appropriately coprecipitated with F-actin. Similarly, GST-dm3 coprecipitated with F-actin, but GST-dm1 and GST-dm2 did not. Therefore we conclude that only domain 3 of *Tetrahymena* eEF1A binds to F-actin.



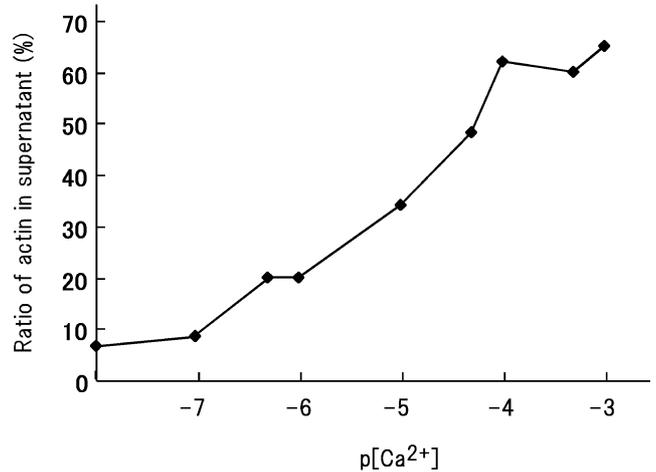
**Fig. 4.** Binding of GST-dm1, -2, and -3 to recombinant *Tetrahymena* CaM at lower  $\text{Ca}^{2+}$  concentrations. Assays were performed in MES buffer containing  $1 \mu\text{M}$   $\text{CaCl}_2$  (A) and  $100 \text{ nM}$   $\text{CaCl}_2$  (B). The supernatants (S) and pellets of beads (P) from the GST pull-down assays were analyzed by SDS-PAGE. Bands corresponding to GST-dm1, GST-dm2, GST-dm3, and CaM are shown by arrows. The molecular mass markers are shown on the left. (1) GST-dm1-bound beads and CaM. (2) GST-dm2-bound beads and CaM. (3) GST-dm3-bound beads and CaM.

### CaM-binding domains in *Tetrahymena* eEF1A

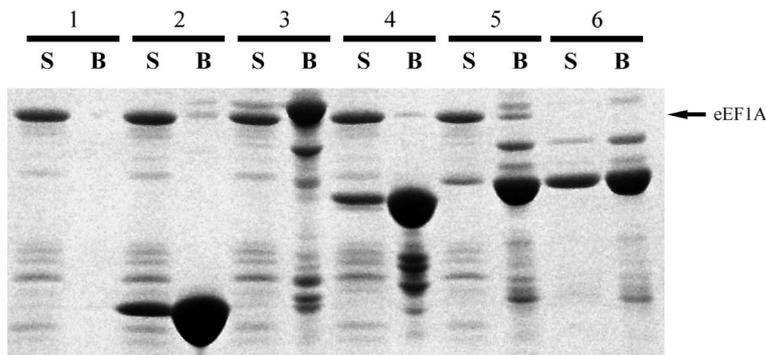
Using the Calmodulin Target Database, we evaluated putative CaM binding sites in *Tetrahymena* eEF1A and found that amino acids 301 through 312 and 414 through 425 each had a score of 9 (Fig. 3A). Therefore it seemed likely that both domain 2 (encoding amino acids 220 through 318) and domain 3 (encoding amino acids 319 through 435) would bind CaM, whereas domain 1 (encoding amino acids 1 through 219) would not.

To identify the CaM-binding domains of *Tetrahymena* eEF1A, we performed GST pull-down assays with GST-dm1, -2, and -3 and recombinant *Tetrahymena* CaM (Fig. 3B, C). GST-dm1, -2, and -3 each bound to recombinant *Tetrahymena* CaM in MES buffer containing  $1 \text{ mM}$   $\text{CaCl}_2$  but not in MES containing  $1 \text{ mM}$  EGTA. Therefore, all three domains of *Tetrahymena* eEF1A bind to *Tetrahymena* CaM in a  $\text{Ca}^{2+}$ -dependent manner.

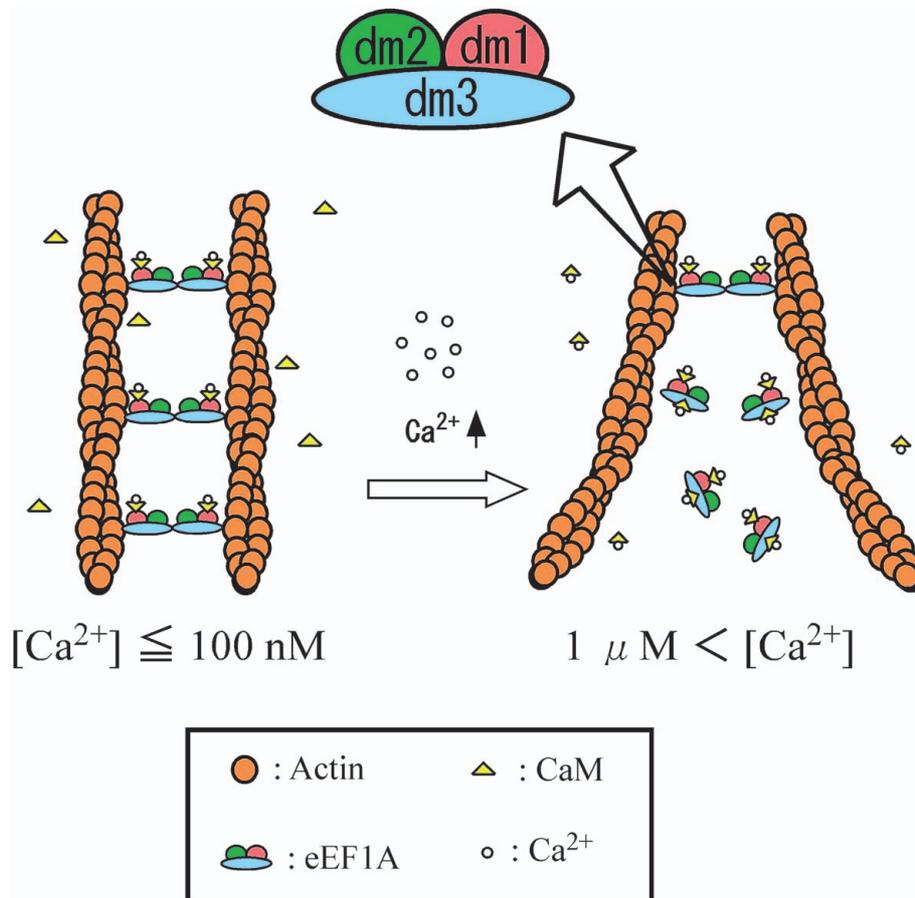
To investigate effects of  $\text{Ca}^{2+}$  concentration on the binding between each domain and CaM, we repeated the GST pull-down assays at lower  $\text{Ca}^{2+}$  concentrations (Fig. 4). Because the concentration of free  $\text{Ca}^{2+}$  in *Tetrahymena* is  $100 \text{ nM}$  or lower, we evaluated binding at  $1 \mu\text{M}$  and  $100 \text{ nM}$   $\text{CaCl}_2$ . In MES buffer containing  $1 \mu\text{M}$   $\text{CaCl}_2$ , GST-dm1 and GST-dm3, but not GST-dm2, bound to recombinant



**Fig. 5.** Inhibition of F-actin bundle formation by CaM at various  $\text{Ca}^{2+}$  concentrations. Cosedimentation assay of  $3 \mu\text{M}$  F-actin,  $3 \mu\text{M}$  eEF1A, and  $3 \mu\text{M}$  CaM was performed at several  $\text{Ca}^{2+}$  concentrations ( $1 \text{ mM}$  EGTA,  $100 \text{ nM}$ ,  $500 \text{ nM}$ ,  $1 \mu\text{M}$ ,  $10 \mu\text{M}$ ,  $50 \mu\text{M}$ ,  $100 \mu\text{M}$ ,  $500 \mu\text{M}$  and  $1 \text{ mM}$   $\text{CaCl}_2$ ). The supernatants and pellets were analyzed by SDS-PAGE, and the amounts of actin in the supernatants and pellets were measured from the strength of the Coomassie Brilliant Blue R-250 staining of the protein bands. The Y axis is the ratio of actin in the supernatant to total actin, and the X axis is the  $\text{Ca}^{2+}$  concentration.



**Fig. 6.** Binding of GST-dm1, -2, and -3 to eEF1A in the absence of  $\text{Ca}^{2+}$ . The supernatants (S) and pellets of beads (P) from GST pull-down assays were analyzed by SDS-PAGE. Bands corresponding to binding of eEF1A to GST-dm3 are shown by an arrow. (1) eEF1A and beads; (2) eEF1A and GST-bound beads; (3) eEF1A and GST-dm1-bound beads; (4) eEF1A and GST-dm2-bound beads; (5) eEF1A and GST-dm3-bound beads; (6) GST-dm3-bound beads alone.



**Fig. 7.** Schematic illustration of the roles of the three domains of *Tetrahymena* eEF1A in F-actin bundling. In the presence of 100 nM or less  $Ca^{2+}$ , eEF1A forms a dimer through domain 3 and subsequently bundles F-actin. In the presence of 1  $\mu\text{M}$  or more  $Ca^{2+}$ ,  $Ca^{2+}$ /CaM binds to domain 3, a moderate amount of eEF1A dimer separates into monomer, and then  $Ca^{2+}$ /CaM/eEF1A monomer complexes dissociate from F-actin. As a result, F-actin bundles are loosened.

*Tetrahymena* CaM. Moreover, in MES buffer containing 100 nM  $CaCl_2$ , GST-dm1 bound to recombinant *Tetrahymena* CaM, and GST-dm3 bound only slightly. These findings indicate that the critical  $Ca^{2+}$  concentration for binding CaM is  $\leq 100$  nM for domain 1, and 100 nM to 1  $\mu\text{M}$  for domain 3.

The F-actin bundling activity of eEF1A is inhibited by  $Ca^{2+}$ /CaM.  $Ca^{2+}$ /CaM binds one of three domains, and converts eEF1A dimer to monomer, and then F-actin bundles are loosened. At the same time, F-actin filaments are dissociated from the F-actin bundles. To examine which domain is involved in the conversion of eEF1A dimer to monomer, we investigated the effect of  $Ca^{2+}$  concentration on dissociation of F-actin bundles by CaM, using F-actin cosedimentation assays (Fig. 5). At 100 nM  $Ca^{2+}$ , F-actin bundles were unaffected. From 500 nM to 1  $\mu\text{M}$   $Ca^{2+}$ , F-actin bundles were loosened, and from 1  $\mu\text{M}$  to 100  $\mu\text{M}$   $Ca^{2+}$ , the amount of F-actin in the supernatant increased. From 100  $\mu\text{M}$  to 1 mM  $Ca^{2+}$ , the amount of F-actin in the supernatant reached equilibrium.

#### Domain for dimer formation in *Tetrahymena* eEF1A

To identify the domain involved in the dimerization of eEF1A, we performed GST pull-down assays with GST-dm1, -2, and -3-bound beads and *Tetrahymena* eEF1A. In

the absence of  $Ca^{2+}$  (Fig. 6) as well as in the presence of 1 mM  $Ca^{2+}$  (data not shown), GST-dm3 bound to *Tetrahymena* eEF1A, but GST-dm1 and GST-dm2 did not.

#### DISCUSSION

This study showed that although all three domains bind to *Tetrahymena* CaM in a  $Ca^{2+}$ -dependent manner, only domain 3 of *Tetrahymena* eEF1A binds to F-actin and is involved in the dimerization of eEF1A. Previously, Bunai *et al.* (2006) demonstrated that eEF1A forms dimers, that dimeric eEF1A bundles F-actin, and that eEF1A monomer binds to but cannot bundle F-actin. Therefore, in *Tetrahymena*, domain 3 has the most pivotal role in the F-actin bundling activity of eEF1A.

Treatment with  $Ca^{2+}$ /CaM separated eEF1A dimers into monomers, loosened F-actin bundles, and then dispersed actin filaments (Bunai *et al.*, 2006). Subsequently,  $Ca^{2+}$ /CaM/eEF1A monomer complexes dissociated from actin filaments (Bunai *et al.*, 2006). Therefore, the  $Ca^{2+}$ /CaM binding site of eEF1A is involved in regulation of the F-actin-bundling activity of eEF1A. All three domains bound to *Tetrahymena* CaM in a  $Ca^{2+}$ -dependent manner (Figs. 3, 4).  $Ca^{2+}$ /CaM inhibition of the F-actin-bundling activity of eEF1A depended on the  $Ca^{2+}$  concentration (Fig. 5). At 100 nM

Ca<sup>2+</sup>, domain 1 could bind to CaM but the F-actin bundles were unaffected, suggesting that binding between domain 1 and CaM has no effect on F-actin bundling by eEF1A. From 500 nM to 1 μM Ca<sup>2+</sup>, domain 3 bound to CaM but domain 2 did not, and F-actin bundles were partially loosened. These findings suggest that domain 3 has a key role in the Ca<sup>2+</sup>/CaM-associated inhibition of F-actin bundle formation. From 1 μM to 100 μM Ca<sup>2+</sup>, the amount of F-actin in the supernatant increased with the Ca<sup>2+</sup> concentration, suggesting that the binding between domain 3 and CaM increases in a Ca<sup>2+</sup>-dependent manner. From 100 μM to 1 mM Ca<sup>2+</sup>, the amount of F-actin in the supernatant reached equilibrium. Therefore, the binding between domain 3 and CaM inhibits F-actin bundle formation by eEF1A.

CaM has four Ca<sup>2+</sup> binding sites, each of which has reasonably high affinity for Ca<sup>2+</sup> ( $K_a \sim 10^{-6} M^{-1}$ ), rendering CaM sensitive to transient increases in Ca<sup>2+</sup> concentration. Stimulation of and subsequent Ca<sup>2+</sup> influx through Ca<sup>2+</sup> channels raise the cellular Ca<sup>2+</sup> concentration from the basal level of ~100 nM to ~10 μM. Because of the precise tuning due to Ca<sup>2+</sup> affinities, only a few CaM molecules are Ca<sup>2+</sup> bound when the cell is in a resting state, but on release of the Ca<sup>2+</sup> signal, all CaM molecules are activated. Cooperative binding is particularly important in this context, as it allows for a tightly controlled “all or nothing” response to changes in Ca<sup>2+</sup> concentration and, consequently, very clean separation of the “off” and “on” states (Nelson and Chazin, 1998).

However, in the contractile ring, F-actin bundles need to be somewhat loose. If the F-actin bundles in the contractile ring were dispersed completely, the contractile ring would break apart and cytokinesis would cease. If the Ca<sup>2+</sup>/CaM/eEF1A-associated looseness of the F-actin bundles was spread over a large range of Ca<sup>2+</sup> concentrations, then the system would be “leaky”—Ca<sup>2+</sup>/CaM would cause some of the eEF1A dimers, which bundle F-actin, to separate into monomers over a large range of Ca<sup>2+</sup> concentrations. Therefore, the weak interaction between domain 3 and Ca<sup>2+</sup>/CaM and the Ca<sup>2+</sup>/CaM-induced partial separation of eEF1A dimer to monomer leads to looseness of the F-actin bundles in the contractile ring.

In several respects, this study supports Bunai's model, but Bunai's model did not explain how the three domains of eEF1A function in F-actin bundling. From our current results—the roles of domain 3 in F-actin binding, eEF1A dimer formation, and Ca<sup>2+</sup>/CaM regulation of eEF1A—we propose a new model of eEF1A-induced F-actin bundling in *Tetrahymena* cells (Fig. 7). In the presence of 100 nM Ca<sup>2+</sup> or less, eEF1A binds to CaM through domain 1, but eEF1A dimerizes through domain 3. Via domain 3, the eEF1A both dimerizes and binds F-actin to form the F-actin bundle. When the Ca<sup>2+</sup> concentration increases from 100 nM Ca<sup>2+</sup> to 1 μM Ca<sup>2+</sup>, Ca<sup>2+</sup>/CaM binds to domain 3 and induces a moderate proportion of eEF1A dimer to revert to monomer, subsequently loosening the F-actin bundles. At the same time, binding of Ca<sup>2+</sup>/CaM to domain 3 induces its separation from F-actin, and the Ca<sup>2+</sup>/CaM/eEF1A monomer complex subsequently dissociates from F-actin.

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