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Tetrahymena Elongation Factor-1 α Binds to Hsp70 Family Proteins

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ABSTRACT—Translation elongation factor 1 α (EF-1 α) catalyzes the GTP-dependent binding of aminoacyl-tRNA to the ribosome. We previously reported that *Tetrahymena* EF-1 α induced bundles of rabbit skeletal muscle F-actin as well as *Tetrahymena* F-actin (Kurasawa *et al.*, (1996) Zool. Sci. 13: 371–375), and that Ca²⁺/calmodulin (CaM) regulated the F-actin-bundling activity of EF-1 α without inhibition of the binding between EF-1 α and F-actin (Kurasawa *et al.*, (1996) J. Biochem. 119: 791–798). In this study, we investigated EF-1 α -binding proteins in *Tetrahymena* using a *Tetrahymena* EF-1 α affinity column. *Tetrahymena* EF-1 α bound directly to 74 kDa, 77 kDa, and 78 kDa proteins, in addition to CaM. The bindings of 74 kDa, 77 kDa, and 78 kDa proteins to *Tetrahymena* EF-1 α were Ca²⁺-independent and ATP-sensitive. The N-terminal amino acid sequence of the 74 kDa protein was similar to those of 70 kDa heat shock protein (hsp70) family.

INTRODUCTION

Translation elongation factor-1 α (EF-1 α) catalyzes the GTP-dependent binding of aminoacyl-tRNAs to their respective mRNA anticodons in the A site of ribosomes in the peptide elongation phase of protein synthesis (Kaziro, 1978; Moldave, 1985; Riis *et al.*, 1990). Several recent reports indicate that EF-1 α appears to have a second role as a regulator of microtubule rearrangements (Kuriyama and Borisy, 1985; Kuriyama *et al.*, 1990; Ohta *et al.*, 1988; Ohta *et al.*, 1990; Shiina *et al.*, 1994). On the other hand, direct and indirect evidence shows that EF-1 α also participates in the regulation of actin cytoskeleton (Demma *et al.*, 1990; Yang *et al.*, 1990; Dharmawardhane *et al.*, 1991; Itano and Hatano, 1991). In previous paper, we demonstrated that *Tetrahymena* EF-1 α could also bind to F-actin and form F-actin bundles (Kurasawa *et al.*, 1996a; Kurasawa *et al.*, 1996b; Numata, 1996). Thus, the EF-1 α is regarded as regulators in the organization of microfilaments as well as microtubules.

A carrot EF-1 α homolog bundled microtubules *in vitro*, and moreover, this bundling was modulated by the addition of Ca²⁺ and CaM together (Ca²⁺/CaM) (Durso and Cry, 1994). Kaur and Ruben demonstrated the direct interaction between CaM and the EF-1 α from *Trypanosoma brucei* (Kaur and Ruben, 1994). Using EF-1 α , actin and CaM separately purified from *Tetrahymena*, we demonstrated that Ca²⁺/CaM di-

rectly bound to EF-1 α and did not effect on the formation of EF-1 α /F-actin complex (Kurasawa *et al.*, 1996a). In addition, Ca²⁺/CaM completely inhibited the formation of F-actin bundles by EF-1 α (Kurasawa *et al.*, 1996a). These findings suggest that the EF-1 α mediates the formation of F-actin bundles and microtubule bundles by a Ca²⁺/CaM-sensitive mechanism. These reports suggest that associations between EF-1 α and cytoskeleton have significance beyond translation.

To ascertain associations between EF-1 α and cytoskeleton, we examined EF-1 α -binding proteins using the *Tetrahymena* EF-1 α affinity column. In this paper, we report direct binding of *Tetrahymena* EF-1 α to 74 kDa, 77 kDa and 78 kDa proteins in Ca²⁺-independent and ATP-sensitive manner, and similarity between an N-terminal amino acid sequence of the 74 kDa protein and those of hsp70 family proteins.

MATERIALS AND METHODS

Cell culture

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

Electrophoresis

SDS-PAGE was performed on a 10 or 15% running gel with a 3% stacking gel according to Laemmli (Laemmli, 1970). Two-dimensional gel electrophoresis was carried out according to the method of Hirabayashi *et al.* (1981). Gels were stained with silver or Coomassie Brilliant Blue R-250.

Preparation of *Tetrahymena* EF-1 α

Tetrahymena EF-1 α was purified by the method described previously (Takeda *et al.*, 1995).

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Preparation of *Tetrahymena* extract

T. pyriformis cells cultivated in 500 ml culture medium for 41 hr were harvested by centrifugation at 1200g for 1 min. The cell pellet was washed twice with cold NKC solution (0.2% NaCl, 0.008% KCl, 0.012% CaCl₂), and divided into two parts. One part was resuspended in 2 volumes of extraction buffer (10 mM MES, 10% glycerol, 1 mM MgCl₂, 5 µg/ml leupeptin, 10 µM *N*α-*p*-tosyl-L-lysine chloro-methyl ketone (TLCK), 3.1 mM NaN₃, pH 7.0) containing 1 mM CaCl₂ (E-Ca buffer). Another part was resuspended in 2 volumes of extraction buffer containing 1 mM EGTA (E-EGTA buffer). Each suspension was sonicated for 4 min at an output setting of 8 using a cell distributor (Ultrasonics Inst., Tomy Seiko, Tokyo, Japan). Each sonicated suspension was centrifuged at 100,000g for 1.5 hr at 0°C. The resultant supernatant prepared in the presence of 1 mM CaCl₂ or 1 mM EGTA was designated as a Ca extract or an EGTA extract, respectively.

Column chromatography

Preparations of *Tetrahymena* EF-1α affinity column and control BSA column were carried out using the HiTrap NHS-activated (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. One ml *Tetrahymena* EF-1α (2 mg/ml) was applied to a HiTrap NHS-activated sepharose column. The column volume was 1 ml. The coupling efficiency was about 2 mg/ml resin. Coupling of BSA to the HiTrap NHS-activated was carried out in the same manner. Column chromatography was carried out in the presence of 1 mM CaCl₂ or 1 mM EGTA. In the presence of 1 mM CaCl₂, each 25 ml of the Ca extract was applied to a *Tetrahymena* EF-1α column or a control BSA column equilibrated with E-Ca buffer at a flow rate of 0.1 ml/min. After loading, each column was rinsed with E-Ca buffer containing 50 mM KCl (EK-Ca buffer) at 0.1 ml/min for 270 min. Then the columns were eluted successively with EK-Ca buffer containing 1 mM ATP, EK-Ca buffer containing 1 mM GTP, E-EGTA buffer containing 50 mM KCl (EK-EGTA buffer), E-Ca buffer containing 150 mM KCl, E-Ca buffer containing 0.4 M KCl and E-Ca buffer containing 1.0 M KCl. On the other hand, in the presence of 1 mM EGTA, 25 ml of the EGTA extract was applied to the *Tetrahymena* EF-1α column equilibrated with E-EGTA buffer. After loading, the column was rinsed with EK-EGTA buffer at 0.1 ml/min for 120 min. Then the column was eluted successively with EK-EGTA buffer containing 1 mM ATP, EK-EGTA buffer containing 1 mM GTP, EK-Ca buffer, E-EGTA buffer containing 150 mM KCl, E-EGTA buffer containing 0.4 M KCl and E-EGTA buffer containing 1.0 M KCl.

Polyclonal antibodies

Rabbit antisera against *T. pyriformis* actin, tubulin and 14 nm filament protein had been previously prepared and characterized (Hirono *et al.*, 1987; Takagi *et al.*, 1991; Numata *et al.*, 1983)

Amino acid sequencing

The 1 mM ATP-eluted fraction was subjected to two-dimensional SDS-PAGE and transferred to a polyvinylidene difluoride filter (Millipore, Bedford, USA). The filter was stained with Coomassie Brilliant Blue R-250. The spot containing only the 74-kD protein was excised from the membrane. N-terminal amino acid sequence of the 74-kD protein was determined by automated Edman degradation with an Applied Biosystems gas-phase sequencer (Foster City, CA, USA).

RESULTS

Tetrahymena EF-1α column-binding proteins from the *Tetrahymena* extract

To investigate a second role of EF-1α as a regulator of cytoskeleton organization, we examine EF-1α-binding proteins in *Tetrahymena*. Aliquots of *T. pyriformis* cell extract, prepared in a low ionic strength buffer containing 1 mM CaCl₂, were

passed through the *Tetrahymena* EF-1α column and the control BSA column. Proteins absorbed to those columns were eluted successively with 1 mM ATP, 1 mM GTP, 1 mM EGTA, 150 mM KCl, 0.4 M KCl, and 1 M KCl, because EF-1α is a GTP-binding protein and the F-actin bundling activity of EF-1α is regulated by Ca²⁺/CaM. Fig. 1A shows SDS-PAGE of each eluate from the *Tetrahymena* EF-1α column. Proteins of apparent molecular weights of 78 kDa, 74 kDa, 60 kDa, 52 kDa and 50 kDa were eluted with 1 mM ATP (ATP eluate) (Fig. 1A, lane 3). The 78 kDa, 60 kDa, 52 kDa and 50 kDa proteins were eluted with 1 mM GTP (GTP eluate) (Fig. 1A, lane 5). In the GTP eluate, the 74 kDa protein was not detected, and the amount of the 78 kDa protein was less than that in the ATP eluate. On the contrary, the amounts of the 52 kDa and 50 kDa proteins in the GTP eluate increased. These results indicate that the bindings of 78 kDa and 74 kDa proteins to *Tetrahymena* EF-1α are ATP-sensitive. A 16.7 kDa protein was a major protein eluted with 1 mM EGTA (Fig. 1A, lanes 7 and 8). Protein of apparent molecular weight of 90 kDa, the 52 kDa and 50 kDa proteins were eluted with 150 mM and 0.4 M KCl (Fig. 1A, lanes 10, 11 and 13).

On the other hand, when *Tetrahymena* cell extract prepared in a low ionic strength buffer containing 1 mM EGTA were passed through the *Tetrahymena* EF-1α column, the 78 kDa and 74 kDa proteins were absorbed to the column, but the 16.7-kD protein was not (data not shown). These results indicate that the bindings of 78 kDa and 74 kDa proteins to *Tetrahymena* EF-1α are Ca²⁺-independent and ATP-sensitive, while the binding between the 16.7 kDa protein and *Tetrahymena* EF-1α is Ca²⁺-dependent. The major proteins seen in the *Tetrahymena* EF-1α column eluate were not significantly absorbed to the control BSA column (Fig. 1B).

Immunoblot analysis of the proteins in the EF-1α column eluate was performed using antibodies against *Tetrahymena* actin, tubulin and 14nm filament protein. We could not identify those proteins in the EF-1α column eluate (data not shown). Since *Tetrahymena* extract was prepared by centrifugation at 100,000 g for 1.5 hr, almost all F-actin, microtubules and 14nm filament might be removed from the extract.

The 74-kD protein is homologous to hsp70 superfamily proteins

The 1 mM ATP-eluted fraction was subjected in two-dimensional gel electrophoresis. As a result, we demonstrated that the fraction contained 78, 77, and 74 kDa proteins (Fig. 2), and that the 78 kDa protein in Fig. 1A, lane 3 contained the 78- and 77 kDa proteins in Fig. 2. The isoelectric points of these proteins were approximately 6.0. The amino acid-sequences of these proteins were then analyzed. Although only N-terminal 25 amino acid-sequence of the 74 kDa protein was determined, the sequence was found to be similar to the predicted N-terminal amino acid-sequences of hsp70 superfamily proteins (Craig *et al.*, 1989; Heschl and Baillie, 1989; Domanico *et al.*, 1993; Rubin *et al.*, 1993; Bardwell and Craig, 1984) (Fig. 3). Previously, we detected *Tetrahymena* hsp70 family proteins in the cell extract of heat-shocked cells using

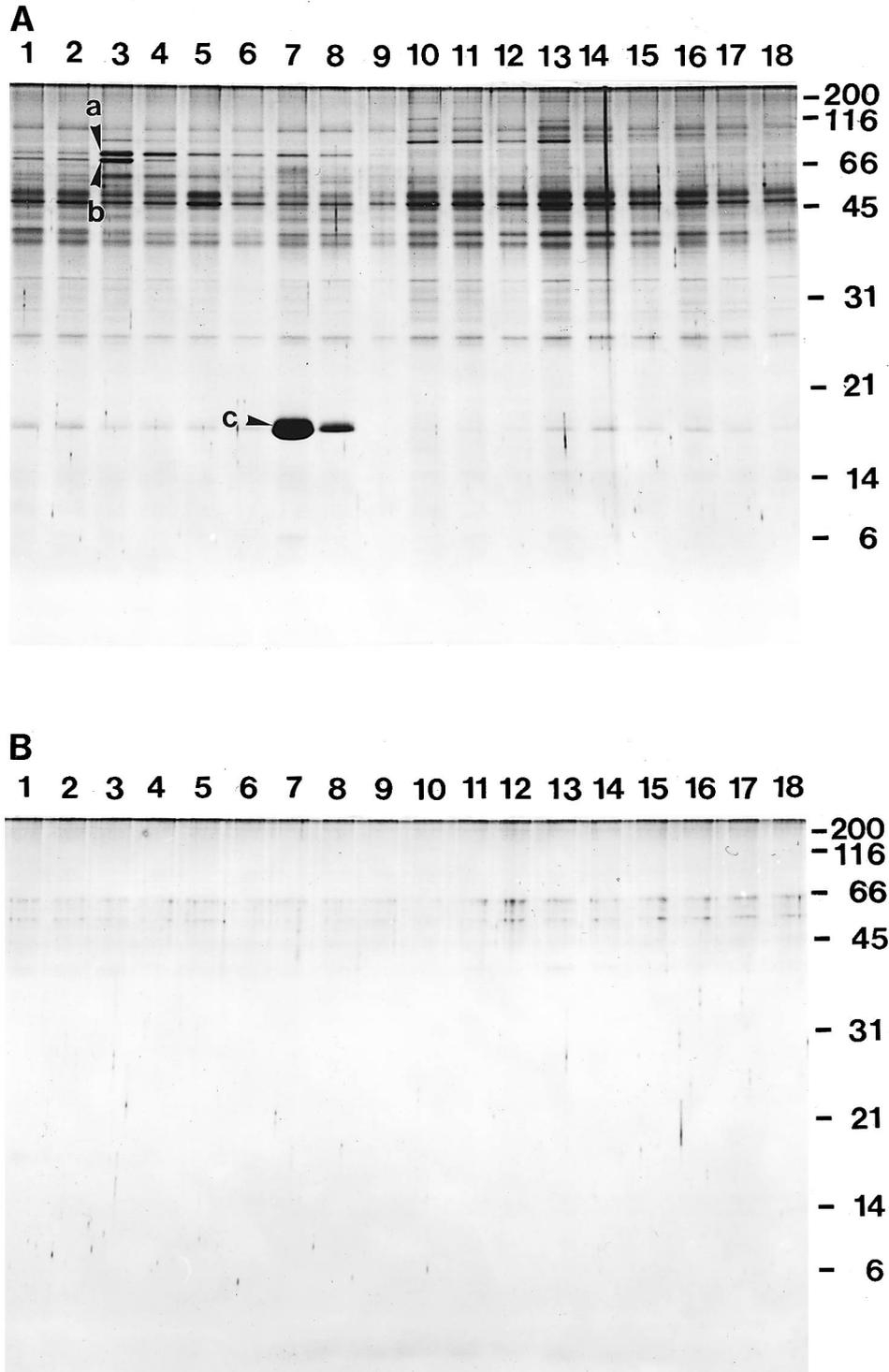


Fig. 1. SDS-PAGE analysis of the proteins eluted from the *Tetrahymena* EF-1 α affinity column which was loaded with a *Tetrahymena* cell extract. The *Tetrahymena* cell extract prepared with E-Ca buffer was applied to the *Tetrahymena* EF-1 α affinity column (**A**) or the control BSA column (**B**). Protein fractions eluted from the *Tetrahymena* EF-1 α affinity column were run on a 15% gel. The gels were silver-stained. Lanes 1 and 2, flow-through fractions; lanes 3 and 4, 1 mM ATP-eluted fractions; lanes 5 and 6, 1 mM GTP-eluted fractions; lanes 7, 8 and 9, 1 mM EGTA-eluted fractions; lanes 10, 11 and 12, 150 mM KCl-eluted fractions; lanes 13, 14, and 15, 0.4 M KCl-eluted fractions; lanes 16, 17 and 18, 1 M KCl-eluted fractions. One mM ATP-eluted fractions from the *Tetrahymena* EF-1 α affinity column (A, lanes 5 and 6) contained 78 kDa and 74 kDa proteins. One mM EGTA-eluted fractions (A, lanes 7 and 8) contained 16.7 kDa protein. These proteins could not bind to the control BSA column (**B**). Arrowheads a, b and c indicate the 78, 74 and 16.7 kDa proteins, respectively. The apparent molecular weights (kDa) are shown on the right.

two-dimensional gel electrophoresis (Takamatsu *et al.*, 1986). The isoelectric points and molecular weights of these hsp70 family proteins almost corresponded to those of the 78, 77, and 74 kDa proteins (data not shown). On the other hand, the 16.7 kDa protein was identified to the *Tetrahymena* CaM, in terms of apparent molecular weight, antigenicity, and a Ca^{2+} -dependent shift in electrophoretic mobility (see other submitted paper).

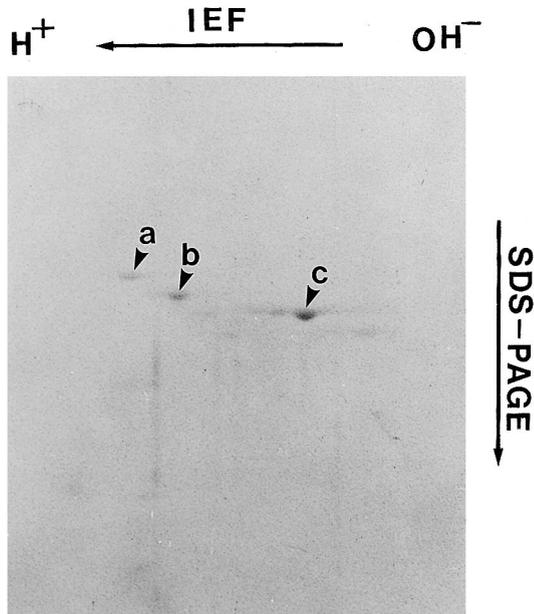


Fig. 2. A two-dimensional gel electrophoresis pattern of the 1 mM ATP fraction eluted from the *Tetrahymena* EF-1 α column. First-dimension is isoelectric focusing (horizontal). Second-dimension SDS-PAGE (vertical) was performed on a 10% running gel. Only the region of the 78 kDa and 74 kDa proteins is shown. Arrowheads a, b, and c indicate the 78 kDa, 77 kDa, and 74 kDa proteins, respectively.

DISCUSSION

In this study, we found that the 74 kDa, 77 kDa and 78 kDa proteins bound to *Tetrahymena* EF-1 α in ATP-sensitive manner (Fig. 1A). The 74 kDa protein was a hsp70 homolog (Fig. 3). This is the first report of direct binding between EF-1 α and hsp70 homolog, although the possibility of association between EF-1 α and hsp70 was reported by Marchesi and Ngo (1993). They isolated multiprotein complexes from supernatants of nocodazole-arrested CHO cells. The complexes, assembled *in vitro* after a 37°C incubation in the presence of ATP or GTP, was composed of equivalent amounts of α -, β - and γ -tubulin, EF-1 α and hsp70. However, tubulin isoforms were not detected to bind the *Tetrahymena* EF-1 α affinity column.

The interaction of the hsp70 family member with its target proteins is transient, because release of the target proteins from the hsp 70 depend on ATP-binding (Palleros *et al.*, 1993). We showed here that the 74 kDa, 77 kDa and 78 kDa proteins were eluted with 1 mM ATP from the EF-1 α affinity column, suggesting that the 77 kDa and 78 kDa proteins may be also the hsp70 family members because they probably have an ATP-binding activity. Williams and Nelsen (1997) have reported that three members of the hsp70 family (hsp72, 73 and 78) and one member of the hsp90 family (hsp82) formed a high molecular mass complex (~ 700 kDa) with tubulin in *Tetrahymena*. Although we could not detect tubulin and hsp90 in eluate from the *Tetrahymena* EF-1 α column, it is possible that the 74 kDa hsp70 homolog, 77 kDa and 78 kDa proteins in the 1 mM ATP-eluted fraction may correspond to hsp72, 73, and 78 respectively and that these members of the hsp70 family may form complexes and function as chaperons.

Hsp70 has been shown to associate with newly synthesized protein during translation and there is ample evidence indicating that hsp70 binds to unfolded proteins (Beckmann *et al.*, 1990). Thus, it appears likely that hsp70 associates with nascent cytosolic proteins to aid in their folding and pre-

(a)	1	A A P G E A I I G I D L G T T N S R V A I M E G S	2 5
(b)	2 6	T K V Q G S V A K	5 0
(c)	4 7	R P A . N D V C . S V K	7 1
(d)	2 7	D K . K G H V C . S K	5 1
(e)	4 9	E A I K G . V V C . V K	7 3
(f)	4 8	G E V K G . V C L . V K	7 2
(g)	1	M G K C D . T	2 2

Fig. 3. N-terminal amino acid sequence of the 74 kDa protein. Comparison of the N-terminal amino acid sequence of the 74 kDa protein with the predicted amino acid sequences of hsp70 family. (a) N-terminal amino acid sequence of the 74 kDa protein, (b) residues 26-50 of *Saccharomyces cerevisiae* hsp70 related protein SSC1 precursor (accession No., A32943) (Craig *et al.*, 1989), (c) residues 47-71 of kidney bean hsp70 (S25005), (d) residues 27-51 of *Caenorhabditis elegans* hsp70F precursor (B32475) (Heschl and Baillie, 1989), (e) residues 49-73 of human mitochondrial hsp70 precursor (B48127) (Domanico *et al.*, 1993), (f) residues 48-72 of *Drosophila melanogaster* heat-shock cognate protein 5 precursor (JN0667) (Rubin *et al.*, 1993), (g) residues 1-22 of *Escherichia coli* dnaK protein (A03311) (Bardwell and Craig, 1984). Dots and underlines indicate identity and similarity, respectively.

vent aberrant protein-protein interactions. The yeast SSB 70 kDa heat shock proteins (Ssb1/2p) have been shown to associated with translating ribosomes (Nelson *et al.*, 1992). This association was disrupted by puromycin, suggesting that Ssb1/2p may bind directly to the nascent polypeptides. The slow growth phenotype of *ssb1 ssb2* mutants was suppressed by increased copy number of a gene encoding a novel EF-1 α -like protein (Nelson *et al.*, 1992). At the present time it is not clear to what degree hsp70 and EF-1 α functions are connected. It is possible that there is relationship between hsp70 and EF-1 α in translation.

Singer and coworker have reported that the majority of poly(A) mRNA in fibroblasts was localized to actin-filament intersections which also contained ribosomes and EF-1 α (Bassel *et al.*, 1994; Bassell and Singer, 1997). The localization of mRNA to intersections suggests that component of the intersection, EF-1 α and α -actinin, are involved in mRNA-cytoskeleton interaction and that intersections may be sites of translation of mRNA. Thus, in translation, EF-1 α brings its multifunctional ability into full play, as the translation elongation factor, the hsp70-binding and the actin cross-linker protein.

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