

# Interactions of eukaryotic elongation factor 2 with actin: a possible link between protein synthetic machinery and cytoskeleton

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**Abstract** Eukaryotic elongation factor 2 (EF-2) was shown to bind to F-actin as assayed by co-sedimentation. In the presence of guanosine-5'-O-(3-thiotriphosphate) (GTPγS) binding was increased fourfold. At saturation level a molar ratio of about 0.12 EF-2 per F-actin (subunit) was observed. Our results suggest a single type of binding site with an apparent dissociation constant of 0.85 μM. The stoichiometry was independent of the filament length, and ADP-ribosylation had no effect on the binding. Experimental data indicated the involvement of SH-groups of both EF-2 and actin in the binding. The interaction EF-2 with F-actin appeared to be inhibited competitively by EF-1α and non-competitively by G-actin.

**Key words:** Elongation factor 2; Actin; Cytoskeleton; Protein synthesis

## 1. Introduction

Actin is an abundant cellular protein which, in filamentous form, plays a central role in the formation of cytoskeletal structures [1]. A large number of proteins with different characteristics and functions interact with this basic component of the cytoskeleton [1,2], regulating its assembly, interactions, and function(s) in the cell.

Recent data indicate that several components of the protein synthetic machinery can also bind to actin microfilaments [3–8]. Among them, elongation factor 1α (EF-1α), previously known as ABP-50 [2,6–10], has turned out to be a major actin-binding protein (ABP). The presence of elongation factor 2 (EF-2) along actin microfilament bundles was shown by immunofluorescence microscopy [11,12]. In the present study, direct evidence for in vitro binding of EF-2 to F-actin is reported together with an investigation of the interaction under different conditions.

## 2. Materials and methods

### 2.1. Materials

EF-2 and EF-1 were prepared as described previously [13]. EF-1 from the hydroxyapatite step was fractionated by chromatography on DEAE-cellulose (DE52 Whatman), and EF-1α, which was not retained by the column, was further purified on phosphocellulose (P11 Whatman) [14]. Both factors were purified to about 90% homogeneity. In order to prepare the nucleotide- and metal ion-free factor, EF-2 was additionally dialyzed, wherever indicated, against dialysis buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 7 mM 2-mercaptoethanol (ME) and 250 mM sucrose) with additional 5 mM EDTA. EF-2 thus

prepared was then dialysed against the dialysis buffer and this material is referred to as EF-2 EDTA.

Diphtheria toxin was a gift from the Refik Saydam Institute in Ankara. All chemicals were purchased from Sigma Chemical Co.; adenine-[<sup>14</sup>C](U)NAD with a specific activity of 518 Ci/mol was from Du Pont-NEN.

Actin was obtained from acetone powder of rabbit skeletal muscle following a polymerization and depolymerization cycle; it was separated from contaminants by chromatography Sephadex G-100. 1 g of acetone powder was resuspended in 20–25 ml G-buffer (5 mM potassium phosphate, pH 7.5, 0.5 mM ATP, 0.1 mM CaCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), and 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and the solution was filtered through a Buchner funnel. G-actin was converted into the filamentous form (F-actin) by bringing the NaCl and MgCl<sub>2</sub> concentrations to 10 mM and 3 mM, respectively (F-buffer). The suspension was first incubated at room temperature for 30 min and then overnight at 4 °C. F-actin was collected by centrifugation at 100,000 × g for 2 h at 14 °C. The pellet resuspended in depolymerization buffer (2 mM potassium phosphate, pH 7.5, 0.5 mM ATP, 0.5 mM DTT, 0.1 mM CaCl<sub>2</sub>, 0.01 mM EDTA and 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) was dialyzed overnight against the same buffer. The suspension was then homogenized with a glass homogenizer, followed by centrifugation at 100,000 × g for 2 h. The supernatant obtained corresponded to the G-actin fraction. F-actin was prepared from this fraction by adjustment anew to F-buffer and centrifugation as indicated above.

### 2.2. Assays

Molar amounts of EF-2 were determined by ADP-ribosylation [15] in the presence of 50 mM Tris-HCl, pH 7.4, 7 mM ME, 5 μM [<sup>14</sup>C]NAD and 120 μg/ml diphtheria toxin.

In each co-sedimentation experiment 200 μl reaction mixtures containing varying amounts of EF-2 (or ADPR-EF-2) and F-actin were incubated at 20 °C as indicated in the figure legends. The buffer used consisted of 0.5 mM GTPγS, 0.5 mM ATP, 3 mM MgCl<sub>2</sub>, 10 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 10 mM ME. After incubation, reaction mixtures were centrifuged at 100,000 × g for 2 h and the protein content of the F-actin pellet was determined [16]. The amount of bound EF-2 was determined by ADP-ribosylation, and where binding of [<sup>14</sup>C]ADPR-EF-2 was assayed by plating aliquots directly on to GF/A (Whatman) filters for scintillation counter measurements. Interaction of EF-2 with F-actin was also investigated by SDS/PAGE analysis [17] of pelleted samples. Proteins were visualised with Coomassie brilliant blue, and autoradiographs of the lanes with samples incubated with diphtheria toxin and [<sup>14</sup>C]NAD were obtained on Kodak X-Omat K films. Finally, EF-2 and actin were also detected in parallel electrophoresis experiments by Western blotting using anti-EF-2 and anti-actin antiserum, respectively [18]. The experiments reported here were repeated at least twice, yielding results with minor deviations (≤ ± 0.05).

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**Abbreviations:** ADPR-EF-2, adenosine diphosphate ribosylated-eukaryotic elongation factor 2; EF-1α, eukaryotic elongation factor 1α; F-actin, filamentous actin; G-actin, globular actin monomer; ABP, actin-binding protein; DT, diphtheria toxin; DTT, dithiothreitol; GTPγS, guanosine-5'-O-(3-thiotriphosphate); NAD, nicotinamide adenine dinucleotide; NEM, N-ethylmaleimide; ME, 2-mercaptoethanol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

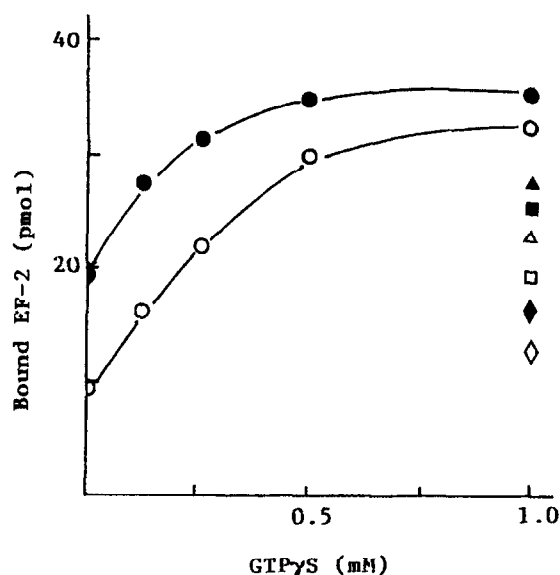


Fig. 1. Effect of GTP $\gamma$ S on binding of EF-2 to F-actin. Incubation (1 h at 20°C), was carried out as described in section 2. In the presence of 4.8 nmol F-actin and 60 pmol EF-2, the concentration of GTP $\gamma$ S was varied between 0 and 1 mM (○—○, ●—●). The effect of other nucleotides at 1 mM concentration are also shown; (△, ▲) GTP, (□, ■) GDP, (◇, ◆) GMP. Closed symbols, untreated EF-2; open symbols, EF-2 EDTA.

### 3. Results

EF-2 was found to bind to F-actin when assayed by co-sedimentation (Fig. 1), and in the presence of GTP $\gamma$ S the amount of bound EF-2 increased twofold. Binding of EF-2 EDTA, the nucleotide- and metal ion-free factor, in the absence of GTP $\gamma$ S, was also investigated: the amount bound to F-actin was reduced by half, but the nucleotide concentration dependence of the interaction followed the same trend and the saturation value was only slightly reduced. The enhancement effect of GTP $\gamma$ S was, in this case, nearly fourfold. GTP and GDP

Table 1

Binding of EF-2 to F-actin under different experimental conditions

Experimental conditions	EF-2 bound (pmol)
<b>Experiment 1</b>	
None (control)	56.5
EF-2/NEM (5 mM, 15 min, 37°C)	27.4
EF-2/NEM (5 mM, 30 min, 37°C)	6.1
EF-2/DT + NAD	51.2
EF-2/preincubation (15 min, 37°C)	48.9
EF-2/preincubation (15 min, 60°C)	45.0
EF-2/ME (0.7 mM, 5 min, 37°C)	71.5
EF-2/ME (3.5 mM, 5 min, 37°C)	75.0
<b>Experiment 2</b>	
None (control)	44.9
EF-2/NEM (5 mM, 30 min, 37°C)	8.8
F-actin/NEM (5 mM, 15 min, 37°C)	24.5
F-actin/NEM (5 mM, 30 min, 37°C)	9.5
<b>Experiment 3</b>	
None (control)	48.0
EF-2/ME (3.5 mM)	60.1
EF-2/ME (7.0 mM)	74.2
EF-2/ME (10.5 mM)	77.0
EF-2/ME (14.0 mM)	61.0
EF-2/ME (17.5 mM)	50.5

Binding of ADPR-EF-2 was assayed after the factor was ADP-ribosylated by incubation for 15 min at 37°C in the presence of 5  $\mu$ M [<sup>14</sup>C]NAD and 120  $\mu$ g/ml diphtheria toxin (DT). To assay alkylation, EF-2 and F-actin were preincubated with NEM and then with 7 mM 2-mercaptoethanol (ME) prior to addition to the reaction mixture. NEM concentration during incubation with ME was 0.7 mM. The effect of freshly added ME was investigated in Experiment 1 after preincubation with EF-2 for indicated periods at 37°C, and in Experiment 3 indicated concentrations of ME were directly added to the reaction mixtures. F-actin amounts used were 4.8 nmol (Experiment 1) and 2.4 nmol (Experiments 2 and 3). The amount of EF-2 was 80 pmol in Experiment 1 and 60 pmol in Experiments 2 and 3. The reaction mixtures were incubated 1 h before centrifugation and binding was assayed as described in section 2.

were less effective than GTP $\gamma$ S, with GDP being the least effective agent. GMP was found to have no effect on the interaction between EF-2 and F-actin.

Effects of different experimental conditions on the binding

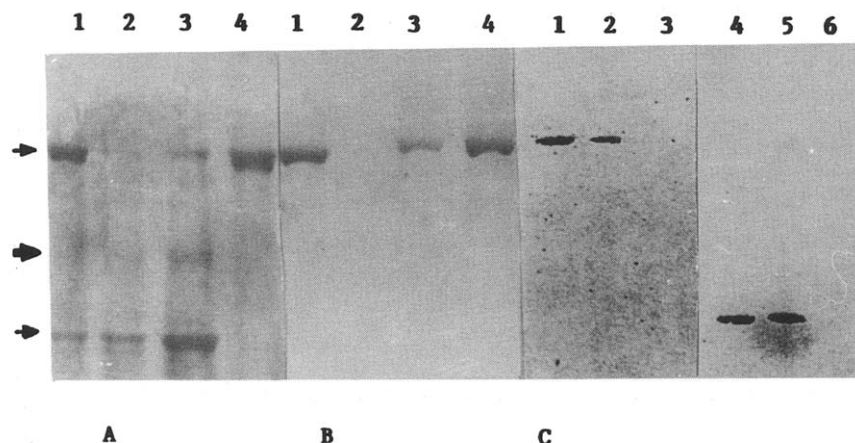


Fig. 2. SDS-PAGE analysis of the EF-2·F-actin complex. F-actin (100  $\mu$ g) incubated with EF-2 (40  $\mu$ g) was subsequently isolated by centrifugation. The pellet was resuspended in 50  $\mu$ l buffer and 40  $\mu$ l thereof was analysed electrophoretically after incubation with DT and [<sup>14</sup>C]NAD [16]. The control EF-2 and the supernatant fraction were subjected to ADP-ribosylation. (A) Lanes 1–4 show supernatant of the EF-2·F-actin complex (40  $\mu$ l), F-actin (20  $\mu$ g), EF-2·F-actin pellet resuspended in F-actin buffer (40  $\mu$ l), and EF-2 (20  $\mu$ g), respectively. (B) Autoradiograph of A. (C) Immunoblot analysis of gels run in parallel. EF-2 antibody staining of EF-2, EF-2·F-actin complex and F-actin are shown in lanes 1–3. Lanes 4–6 are F-actin, EF-2·F-actin complex and EF-2 stained using anti-actin antibody. Arrows indicate the marker proteins; phosphorylase *b* (97 kDa, top band) and ovalbumin (45 kDa, bottom band). The middle arrow indicates the position of diphtheria toxin.

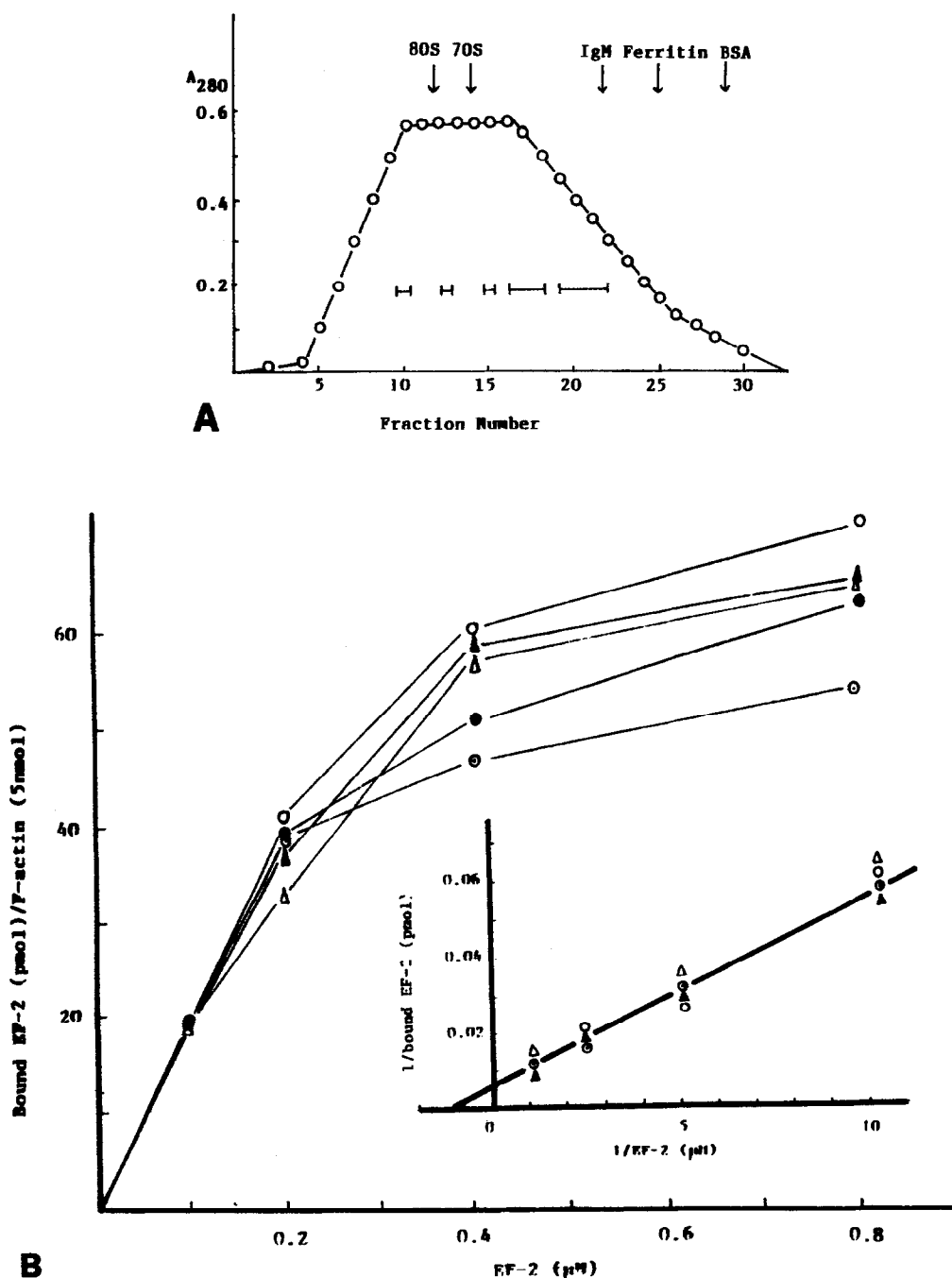


Fig. 3. Size-fractionation of F-actin and binding of EF-2 to F-actin fractions of defined lengths. (A) Fractionation of F-actin according to polymer size on a Sepharose 4B column. The column ( $1 \times 60$  cm), developed with F-buffer, was calibrated using bovine serum albumin (BSA) ( $M_r$  67 kDa), ferritin ( $M_r$  ~450 kDa), IgM-alkaline phosphatase conjugate ( $M_r$  ~900 kDa), *E. coli* 70 S ribosome ( $M_r$  ~3.0 MDa) and rat liver 80 S ribosome ( $M_r$  4.5 MDa). Elution positions of the standards were determined by measurements at  $A_{260}$  and  $A_{280}$ . Elution of IgM-alkaline phosphatase was followed by an assay for alkaline phosphatase activity based on *p*-nitrophenyl-phosphate hydrolysis [19]. F-actin fractions corresponding to about 1, 2, 3, 4 and 5 MDa  $\pm$  250 kDa were pooled as indicated and collected by centrifugation at  $100,000 \times g$  for 6 h. Pellets were resuspended in polymerization buffer for binding experiments. (B) Binding of EF-2 to actin polymers of different length. Varying amounts of EF-2 were incubated with 4.8 nmol fractionated F-actin for 1 h and binding was assayed as described in section 2. Lengths of F-actin fractions were (●—●) 1 MDa, (○—○) 2 MDa, (▲—▲) 3 MDa, (△—△) 4 MDa and (○—○) 5 MDa. Values are given as EF-2 bound per F-actin. (Inset B) Evaluation of the data given in B by double reciprocal plot as described in [20].

of EF-2 to F-actin are presented in Table 1. As can be seen from the results of Experiments 1 and 2, incubations of EF-2 with NEM resulted in a decrease of 90% in the amount of bound EF-2. Similarly, incubation of F-actin with NEM reduced the binding by about 80%. Results of Experiment 3 showed that

freshly added ME enhanced the extent of interaction nearly twofold at an optimum concentration of 10 mM. These findings collectively suggest an involvement of SH-groups of both EF-2 and F-actin in the observed interaction. A preincubation of EF-2 at 37 or 60°C had only a slight inhibitory effect, and

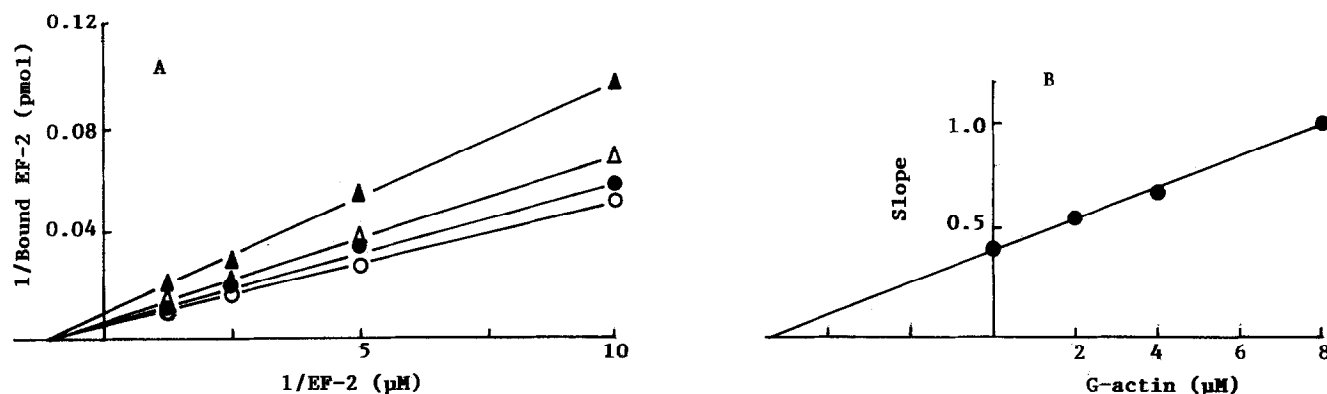


Fig. 4. Effect of G-actin on binding of EF-2 to F-actin. Varying amounts of EF-2 were incubated (6 h at 20°C) with F-actin (2.4 nmol) in the presence of different amounts of G-actin. Binding was assayed as described in section 2. (A) Evaluation of the binding data by a double reciprocal plot. G-actin concentrations were; (○—○) 0 μM, (●—●) 2 μM, (Δ—Δ) 4 μM, (▲—▲) 8 μM. (B) Plot of the slopes obtained from A as a function of G-actin concentrations.

ADP-ribosylation of EF-2 did not affect the binding (Experiment 1). A minor decrease observed in the latter appears to be due to preincubation of EF-2 at 37°C.

The binding was also confirmed by SDS-PAGE analysis of F-actin, collected by ultracentrifugation after incubation with EF-2 (Fig. 2). A 95 kDa polypeptide, which corresponded to the reference EF-2 band, was hereby observed in the F-actin pellet fraction. Autoradiographic analysis following [<sup>14</sup>C]ADP-ribosylation, as well as immunoblot analysis using anti-EF-2 serum, supported this finding.

In early experiments the stoichiometry of the binding was found to be low. This raised the question of the nature and the site of binding on F-actin. In order to gain an insight into these features, experiments were repeated with polymer fractions of defined lengths obtained by chromatography on Sepharose 4B (Fig. 3). The amount of EF-2 bound to F-actin subunit was not affected by polymer size, indicating binding along the filament.

The stoichiometry of binding was found to be 0.12 EF-2 bound per actin subunit. There was a single type of binding with an apparent dissociation constant of about 0.85 μM

(Figs. 4 and 5). G-actin inhibited binding of EF-2 to F-actin, suggesting an interaction of the factor with the monomer as well. The inhibition was non-competitive, as indicated by the double reciprocal plot of the binding data. A plot of the slope values vs. G-actin concentrations yielded a  $K_i$  of 5.2 μM (Fig. 4). EF-1α caused, in turn, a competitive inhibition of the binding of EF-2, which may imply identical (or overlapping) binding sites on F-actin. A  $K_i$  value of around 0.21 μM was found for EF-1α (Fig. 5).

#### 4. Discussion

The present investigation indicates that EF-2 is, like EF-1α, an actin-binding protein, with an affinity comparable to that of several other actin-binding proteins [1,21,22]. The stoichiometry is independent of the size of the filament, suggesting binding of the factor along the filament. These results are supported by a previous report that EF-2 is detected by indirect immunofluorescence microscopy along actin microfilament bundles [12].

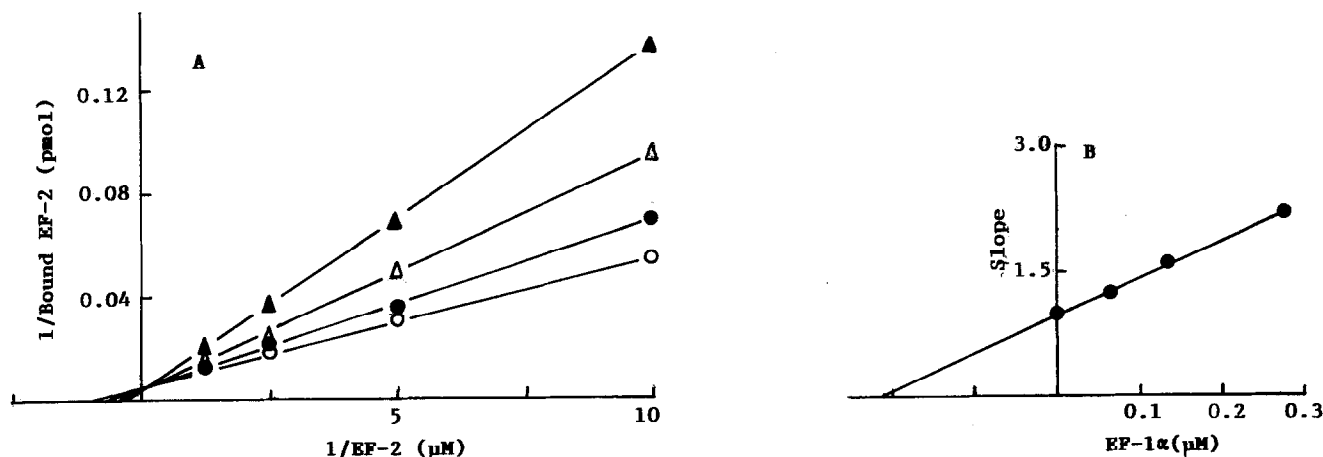


Fig. 5. Competitive inhibition of binding of EF-2 to F-actin by EF-1α. Varying amounts of EF-2 were incubated (6 h at 20°C) with F-actin (2.4 nmol) in the presence of different amounts of EF-1α. Binding was assayed as described in section 2. (A) Evaluation of the binding data by a double reciprocal plot. EF-1α concentrations were; (○—○) 0 μM, (●—●) 0.065 μM, (Δ—Δ) 0.13 μM, (▲—▲) 0.26 μM. (B) Plot of the slopes obtained from A as a function of EF-1α concentrations.

The physiological significance of the interaction of EF-2 with actin remains to be established. However, the results presented here and the established role of EF-1 $\alpha$  as a major actin-binding protein [2,10] strongly suggest a possible regulatory link between the protein synthesizing machinery and cytoskeleton. The much higher cellular content of actin [23] is possibly balanced by the higher affinity of EF-2 in its ribosomal interactions [24,25]. In this context, an about twofold excess of EF-2 over ribosomes in the cell deserves special consideration [26–28], and our own unpublished results).

EF-1 $\alpha$  competitively inhibits the binding of EF-2, suggesting common or overlapping binding sites on F-actin. Mutual exclusion of these factors on the ribosome is well known [29], but its significance in the case of F-actin remains obscure. Immunofluorescence light microscopy, on the other hand, reveals important differences with regard to the cellular compartmentalization of EF-1 $\alpha$  and EF-2. Whereas the former is localized in the cell cortex and filopodia [8], EF-2 is found mainly in the perinuclear region [11,12], and our own unpublished results). Some actin-binding proteins may be responsible for the different distribution of these two factors in the cell.

GTP $\gamma$ S enhances the binding of EF-2 to F-actin by about fourfold. GTP and GDP are, in turn, less effective in promoting binding. The two latter nucleotides appear to have no effect on the binding of EF-1 $\alpha$  to F-actin [8]. EF-1 $\alpha$  is reported to exhibit a moderate affinity for F-actin, with a  $K_d$  of 2.1  $\mu$ M [7]. This value differs from the  $K_d$  value reported for EF-1 $\alpha$  in the present study. The presence of GTP $\gamma$ S in our competition assays may account for the increased affinity of EF-1 $\alpha$  for F-actin.

These results pose the question of how the post-synthetic modifications of EF-2 (i.e. ADP-ribosylation and phosphorylation [30]) may affect the interactions of EF-2 with the cytoskeleton. In this context ADP-ribosylation may be of particular interest since diphtheria toxin is known to give rise to fragmentation of myocardial fibrils. This effect may gain new meaning in the light of the reported interaction. A function of EF-2 in the organization of cytoskeleton may well be additionally affected by ADP-ribosylation. That ADP-ribosylation fails to inhibit the interaction does not yet exclude such a hypothetical function.

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