The Effect of F-actin on the Binding and Hydrolysis of Guanine Nucleotide by Dictyostelium Elongation Factor 1A*

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Indirect evidence implicates actin as a cofactor in eukaryotic protein synthesis. The present study directly examines the effects of F-actin on the biochemical properties of eukaryotic elongation factor 1A (eEF1A, formerly EF1α), a major actin-binding protein. The basal mechanism of eEF1A alone is determined under physiological conditions with the critical finding that glycerol and guanine nucleotide are required to prevent protein aggregation and loss of enzymatic activity. The dissociation constants (KD) for GDP and GTP are 2.5 μM and 0.6 μM, respectively, and the kat of GTP hydrolysis is 1.0 × 10⁻³ s⁻¹. When eEF1A binds to F-actin, there is a 7-fold decrease in the affinity for guanine nucleotide and an increase of 35% in the rate of GTP hydrolysis. Based upon our results and the relevant cellular concentrations, the predominant form of cellular eEF1A is calculated to be GTP-eEF1A:F-actin. We conclude that F-actin does not significantly modulate the basal enzymatic properties of eEF1A; however, actin may still influence protein synthesis by sequestering GTP-eEF1A away from interactions with its known translational ligands, e.g. aminoacyl-tRNA and ribosomes.

Actin has been implicated as a co-factor in eukaryotic protein synthesis (reviewed in Refs. 1–3). Early cell ultrastructural studies revealed an association between components of the translational machinery and the actin cytoskeleton (4, 5), and functional studies showed that mRNAs associated with detergent-insoluble material, presumably the cytoskeleton, are translated more efficiently (6). Subsequently, it has been shown that virtually all of the protein components of translation associate with the cytoskeleton in situ (7–12); however, no direct effect of the cytoskeleton on the specific activity of any of these co-factors has been demonstrated. Eukaryotic elongation factor 1A (eEF1A, formerly EF1α) is the most abundant G protein in cells, comprising 1–5% of total cell protein (14–16). The principal role of eEF1A during protein synthesis is to bind aminoacyl-tRNA to the A-site of the ribosome by a GTP-dependent mechanism (17). Following the hydrolysis of GTP, aminoacyl-tRNA is incorporated into the growing polypeptide, and GDP:eEF1A is released from the ribosome. The exchange of bound GDP for free GTP regenerates the capacity of eEF1A to bind another aminoacyl-tRNA and to participate in further cycles of elongation. Therefore, the affinity of eEF1A for GDP and GTP and the kinetics of GTP hydrolysis are potential targets for ligands operating through eEF1A to regulate protein synthesis. For example, the rate of GDP/GTP exchange by eEF1A in vitro is too slow to support the measured rate of polypeptide elongation in vivo; therefore, the need for a nucleotide exchange factor has been postulated (18, 19).

eEF1A was identified in a screen for actin binding proteins in Dictyostelium and was shown to bind both monomeric and filamentous actin (F-actin) in vitro and in situ (20–23). Due to the striking similarity of eEF1A primary sequences among species, it is not surprising that eEF1As purified from many sources display similar actin binding activities in vitro (24). Thus, it has been concluded that actin binding is a well conserved property of all eEF1As. Since actin and eEF1A are very abundant proteins in cells and interact with good affinity (21, 23, 26), the physiological relevance of their interaction deserves investigation.

An unusual characteristic of eEF1A is the ability following cell stimulation to cross-link actin filaments into bundles whose properties are unique compared with other actin-containing structures (16, 21, 23, 25–27). Observations that stimulation of many cell types induces an increase in the rate of protein synthesis and a redistribution of specific mRNAs and eEF1A to the F-actin-rich leading edge of lamellipods suggest a link between the protein synthetic activity of eEF1A and its novel bundling of actin filaments at the leading edge (16, 26, 28, 29). Do the eEF1A:actin bundles merely serve as a supporting scaffold upon which translation of these targeted mRNAs occurs, or do actin filaments modulate the enzymatic activity of the cofactors involved in protein synthesis?

The present study directly examines the effect of F-actin on the biochemical properties of eEF1A that are relevant to protein synthesis (guanine nucleotide binding and GTP hydrolysis), and a model for how localized protein synthesis may be influenced by the interaction of eEF1A with actin is proposed.

EXPERIMENTAL PROCEDURES

All reagents were purchased from Sigma unless otherwise noted. Purification of Protein—eEF1A was isolated from Dictyostelium discoideum strain AX3, as described previously (26), except GDP (50 μM; [eEF1A] + 20 Kᵦ) was included in all buffers. The addition of guanine nucleotide was critical to stabilize the enzymatic activity of the purified eEF1A (see “Results”). When necessary, nucleotide-free eEF1A was prepared by extensive dialysis against a buffer containing 30% glycerol. Under these glycerol-containing, nucleotide-free conditions, the protein was stable for 1 or 2 days at 4 °C.

The protein concentration of purified eEF1A was determined spec...
trophometrically at 280 nm using an extinction coefficient (43,400 M⁻¹ cm⁻¹) calculated from the deduced amino acid sequence of the Dictyostelium eEF1A cDNA (10). The relative accuracy (±10%) of this extinction coefficient was confirmed independently by a total amino acid composition analysis of purified eEF1A (data not shown). In addition, Bradford protein quantitation assays using bovine serum albumin as standard were within 10% of the value obtained spectrophotometrically on the same samples (data not shown). Dictyostelium actin was purified and stored as described elsewhere (30).

**Fluorescent Nucleotide Binding Assays**—The N-methylanthraniloyl derivative of 2'-deoxy-GDP (mant-GDP) was prepared according to the method of Yang and Leyh (31). N-Methylisatoic anhydride was purchased from Molecular Probes (Eugene OR). We chose to synthesize the 2'-deoxy compound to obviate complications in experimental interpretation of changes in quantum yield caused by the spontaneous isomerization of the mann moieties between the 2'- and 3'-positions of the ribose ring (32). The authenticity of the mant-GDP compound was assessed spectrophotometrically, and its purity was determined to be greater than 95% by high pressure liquid chromatography ion exchange chromatography.

**RESULTS**

**Guanine Nucleotide and Glycerol Are Required to Stabilize eEF1A**—The binding and hydrolysis of guanine nucleotides are cardinal aspects of eEF1A function and are central to its physiological roles. A limitation of previous determinations of nucleotide binding constants for eEF1A is the reliance on membrane filtration assays to separate enzyme-nucleotide complexes from free nucleotides (see Table I). Due to the extensive washing required, the use of membrane filtration to detect binding interactions is an inherently inaccurate approach for proteins with dissociation constants above 20 nM and therefore may be unsuitable for eEF1A (37). To assess the nucleotide binding activity of Dictyostelium eEF1A, we synthesized mant-GDP, a fluorescent analog of GDP whose quantum yields changes during interaction with guanine nucleotide-specific enzymes (38). This and related compounds have proven to be excellent mimics of native nucleotides in mechanistic studies of other GTPases (32, 39, 40). In agreement with others, we find that purification and storage of eEF1A in glycerol is necessary to stabilize its nucleotide binding activity (41–44); however, the further addition of guanine nucleotide is critical in obtaining fully active protein.

Fig. 1A shows pooled data from three separate mant-GDP binding titrations using eEF1A purified with buffers containing glycerol and GDP at saturating concentrations (see “Experimental Procedures”). All nucleotide binding experiments were performed at 22 ± 1 °C, the optimum temperature for the normal growth of Dictyostelium. The experimental data are well fit by a single binding site model in which each molecule of eEF1A binds a single molecule of mant-GDP (33). Analysis of the data using a hyperbolic nonlinear least squares fitting algorithm indicates a $K_d$ of 2.64 ± 0.16 μM with a binding
stoichiometry of 1.0 ± 0.05. Recasting the binding data in the form of a Scatchard plot results in the linear profile expected for one independent binding site (Fig. 1B).

Surprisingly, the mant-GDP binding properties of eEF1A purified without GDP in the running buffers are very different: the stoichiometry of binding is 0.6, and Scatchard plots display the convex profile indicative of positive cooperativity (45) (data not shown). Since eEF1A possesses only one nucleotide binding site, cooperative nucleotide binding indicates protein-protein interaction. Moreover, the nucleotide binding properties of eEF1A isolated in the presence of GDP irreversibly convert to lower stoichiometries and positive cooperativity after dialysis against nucleotide-free, glycerol-containing buffers (data not shown). Thus, it would appear that glycerol and guanine nucleotides are critical to prevent protein aggregation in vitro.

The Affinity of eEF1A for Native GDP—A competitive binding experiment using mant-GDP was designed to determine the affinity of eEF1A for native GDP. Increasing concentrations of GDP were titrated into a mixture containing eEF1A (3.0 μM) and mant-GDP (2.0 μM), and changes in the fluorescence intensity were monitored (Fig. 1C). The K<sub>D</sub> for GDP was determined by fitting the resulting intensity profile using the appropriate root of a third-order polynomial (31). The best fit of the experimental data was obtained with a K<sub>D</sub> for GDP of 2.5 μM, indicating similar affinities of eEF1A for mant-GDP and GDP. Similarities in the binding of mant-derivative and native nucleotides were also demonstrated for ras (32) and EF1A (EF-Tu) (39); thus, our data further confirm mant-GDP as a good mimic for native GDP in nucleotide binding studies.

Pre-steady-state Kinetics of GTP Hydrolysis—The kinetics of GTP hydrolysis and product release are proposed to regulate elongation; the rate of hydrolysis may influence the fidelity of aminoacyl-tRNA:mRNA codon-anticodon pairing (46), and the rate of GDP/GTP exchange may limit the availability of active GTP:eEF1A for subsequent ternary complex formation (19). Since product release has been shown to be rate-limiting for other GTPases, an experiment was designed to detect the formation of a single enzyme equivalent of product. Under these conditions, if product release is rate-limiting, a burst of GDP formation will be observed.

Fig. 2A shows a typical progress curve with eEF1A (1.0 μM) under saturating concentrations of GTP (20 μM; 20 × K<sub>m</sub>). The hydrolysis products 32P<sub>γ</sub> and GDP were separated by thin layer chromatography and quantitated by a two-dimensional scanning radioactivity detector. Two important observations can be made from this experiment. First, there is no evident burst of GDP formation (although under these assay conditions a tiny burst will not be detected). This result indicates that product release is not rate-limiting for eEF1A, a conclusion further borne out by the linearity of the hydrolysis reaction over multiple enzyme turnovers. These results are in contrast to prokaryotic EF1A (EF-Tu), where GTP hydrolysis slows dramatically after one enzyme equivalent of product has formed (47). Second, the measured rate constant for GTP hydrolysis (k<sub>cat</sub>), 1 × 10<sup>−3</sup> s<sup>−1</sup>, is 3–6-fold less than those reported for other eEF1As (see Table I). The source of this latter difference is probably related to glycerol and enzyme stability; when we omit glycerol, k<sub>cat</sub> falls to levels comparable with the other eEF1As (∼0.2 × 10<sup>−3</sup> s<sup>−1</sup>).

The Kinetic Constants for GTP Hydrolysis—Initial rate studies were performed to determine the kinetic constants for the hydrolysis of GTP by eEF1A. The rates were determined under initial velocity conditions; the reactions were allowed to proceed to less than 10% of their end points. The concentration of GTP substrate was varied in even increments in reciprocal space from 0.1 to 25 μM. A double reciprocal plot of the data is shown in Fig. 2B. The data were fit using the weighted least squares program hypero developed by Cleland (35). The kinetic constants are as follows: K<sub>m</sub> = 0.60 ± 0.12 μM; k<sub>cat</sub> = 1.07 ± 0.07 × 10<sup>−3</sup> s<sup>−1</sup>.

Nucleotide Binding Is at Equilibrium during GTP Hydrolysis—If nucleotide association and dissociation are fast compared with cleavage, the binding reaction is near equilibrium, and the Michaelis constant (K<sub>m</sub>) provides a measure of thermodynamic affinity; i.e. K<sub>m</sub> = K<sub>D</sub>. The physical basis for this equivalence can be appreciated by considering the case where GTP is not hydrolyzed; given sufficient time, the binding steps will equilibrate. If GTP hydrolysis were then allowed to occur at a rate that is insignificant compared with the adsorption and

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**Fig. 1. Guanine nucleotide binding properties of eEF1A isolated and stored in the presence of native GDP.** The Langmuir isotherm (A) and the corresponding Scatchard plot (B) of mant-2'-deoxy-GDP binding are shown. The concentration of mant-GDP was fixed at 2.0 μM, while the concentration of nucleotide-free eEF1A was varied as indicated. Nucleotide-free eEF1A was obtained by extensive dialysis against buffer lacking nucleotide. Loss of nucleotide was confirmed by comparing the absorption spectra pre- and postdialysis. C, binding competition between mant-GDP and native GDP. Changes in the fluorescence intensity of mant-GDP (2.0 μM) incubated with eEF1A (3.0 μM) were monitored after equilibration with the indicated concentrations of native GDP. Buffer conditions were 20 mm Pipes, pH 6.5, 50 mm KCl, 2 mm MgCl<sub>2</sub>, 1 mm ATP, 0.2 mm dithiothreitol, and 30% glycerol. r represents the ratio of eEF1A bound to total mant-GDP; E<sub>i</sub> represents the concentration of free eEF1A. The solid lines indicate the best fits to the experimental data (■) generated by curve-fitting algorithms (see “Experimental Procedures” and Ref. 22).
desorption of GTP, the binding steps would remain essentially at equilibrium. In this case, the double-reciprocal plot (Fig. 2B) represents an equilibrium titration of the enzyme, and the value of $K_d$ obtained provides an excellent approximation of the $K_d$.

A two-stage mixing experiment was designed to determine if exchange between the eEF1A-bound and free pools of GTP is fast compared with product formation (48). To perform this experiment, eEF1A is first mixed with a saturating concentration of $[\gamma^{32}P]GTP$ (10 $\mu$M, 10 $\times$ $K_d$). This solution is quickly diluted 5% by hand with a concentrated solution of unlabeled GTP, producing a final unlabeled GTP concentration 100 times that of the labeled GTP. If the exchange of enzyme-bound and free nucleotide is slow compared with hydrolysis, a fraction of the eEF1A-bound $[\gamma^{32}P]GTP$, present at $t = 0$ of mixing, will be converted to product. If, however, GTP exchanges quickly compared with hydrolysis, the eEF1A-bound labeled GTP will be displaced by the exogenous unlabeled GTP, and no further $^{32}P_i$ will be produced.

As can be seen from Fig. 2C, the initial hydrolysis reaction containing only eEF1A and labeled GTP proceeds linearly for $\sim$1.5 enzyme turnovers. After the addition of the unlabeled GTP (arrow), the further production of $^{32}P_i$ ceases within the 12 s required for mixing and to quench the next sample. For the remainder of the experiment, the level of $^{32}P_i$ remains constant.

This result indicates that the added GTP exchanges rapidly with the $[\gamma^{32}P]GTP$ bound to eEF1A; therefore, the $K_d$ of GTP hydrolysis by eEF1A (0.6 $\mu$M) closely approximates the $K_d$ of eEF1A for GTP. We also conclude from this experiment that the rate of GTP dissociation for Dictyostelium eEF1A must be faster than the rate of GTP hydrolysis; i.e. $>1 \times 10^{-3}$ s$^{-1}$.

**Guanine Nucleotides Reduce the Affinity of eEF1A for F-actin**—G proteins are proposed to serve as molecular switches for many cellular processes (15). This idea comes from observations that G proteins in the GDP-bound state “turn on” their associated metabolic pathways compared with “shutting off” the same reactions when in the GDP-bound state. For example, EF1A (formerly EF-Tu), the prokaryotic homolog of eEF1A, during the transition from the GDP- to GTP-bound states, undergoes a dramatic conformational change that increases the affinity for aminoacyl-tRNA and enables participation in protein synthesis (49, 50). Due to the proposed structural similarity between eEF1A and EF1A (24), it is expected that eEF1A displays similar nucleotide-dependent conformational dynamics.

The in vivo interaction of eEF1A with actin presumably is affected by a molecular switch associated with the intracellular signaling pathways activated by cell stimulation (16, 21, 27). For this reason, it was of interest to determine if the nucleotide state of eEF1A influences the interaction with F-actin. Varying concentrations of eEF1A were incubated with *Dictyostelium* actin (2.0 $\mu$M) and 1 mM GDP or GTP at 22 $^\circ$C, and actin filaments and eEF1A bound to them were separated from unpolymerized actin and free eEF1A by high speed centrifugation (see “Experimental Procedures”). In the case of experiments using GTP, the calculated maximum concentration of GDP produced by the hydrolysis of GTP by eEF1A during the 18-h incubation would be less than 0.1 mM, an amount that will not significantly compete with the remaining 0.9 mM GTP for binding to eEF1A (see Table III).

As shown in Fig. 3 and Table II, both GDP and GTP weaken the binding of eEF1A to F-actin. At the pH optimum for actin binding (26), the $K_d$ of nucleotide-free eEF1A for F-actin, 0.17 ± 0.002 $\mu$M (top panel), is increased to 1.32 ± 0.06 $\mu$M by GDP (middle panel) and to 1.21 ± 0.1 $\mu$M by GTP (lower panel). The $K_d$ of nucleotide-free eEF1A for actin is in good agreement with our previous observations (26), while the difference between the two $K_d$ values in the presence of GDP and GTP is not statistically significant. Thus, guanine nucleotides decrease the affinity of eEF1A for F-actin by 7–7.5-fold. The differences between the apparent stoichiometries of binding (number of bound eEF1As per actin monomer) for the three nucleotide conditions are not statistically significant: nucleotide-free, 1.56 ± 0.1; GDP, 1.45 ± 0.13; and GTP, 1.78 ± 0.4 (see Table II).

A separate experiment was performed to measure directly the stoichiometry of eEF1A binding to F-actin under saturating conditions. Actin (2.0 $\mu$M) was co-polymerized as above with a saturating amount of eEF1A (10 $\mu$M; 10 $\times$ $K_d$). After high speed centrifugation, the F-actin and eEF1A contained in the pellets were separated by SDS-polyacrylamide gel electrophoresis, and the amounts were quantitated by scanning densitometry.
F-actin and the Enzymatic Properties of eEF1A

FIG. 3. The effect of guanine nucleotides on the binding interaction between eEF1A and F-actin. Using the buffer conditions of Fig. 1, varying concentrations of nucleotide-free eEF1A were incubated with actin (2.0 \mu M) in the absence (Free) or presence of 1 mM GDP or GTP. Following high speed centrifugation, the amounts of bound eEF1A and F-actin were calculated from the differences between the total and free concentrations as measured by densitometry of SDS-polyacrylamide gel electrophoresis gels. The data are presented in the form of double reciprocal plots. r represents mol of bound eEF1A/mol of total F-actin.

TABLE II

| Nucleotide bound   | \( K_d \) (\mu M) | Stoichiometry\(^a\) | Stoichiometry\(^b\) |
|--------------------|------------------|----------------------|----------------------|
| None               | 0.17 (0.002)     | 1.56 (0.1)           | 1.42 (0.05)          |
| GDP                | 1.32 (0.06)      | 1.45 (0.13)          | 1.46 (0.04)          |
| GTP                | 1.21 (0.1)       | 1.78 (0.4)           | 1.52 (0.02)          |

\(^a\) Estimated from Fig. 1.
\(^b\) Determined directly (see “Experimental Procedures”).

The results are shown in Table II. The differences between the molar ratios of eEF1A bound to F-actin again are not statistically significant: nucleotide-free, 1.42 \pm 0.05; GDP, 1.46 \pm 0.04; and GTP, 1.52 \pm 0.02. These stoichiometries agree with the values obtained from the double reciprocal plots (Fig. 3 and Table II) but contain less variability in the S.E. values.

Thermodynamic Predictions for the Affinity of eEF1A for F-actin and Guanine Nucleotides—For a closed system, the difference in chemical potential between any two states of the system is independent of the path taken between these two points. This scheme is diagrammed in Fig. 4 for the formation of GDP-eEF1A-F-actin or GTP-eEF1A-F-actin complexes. The energetic consequence of this first law of thermodynamics for this allosteric system is that a decrease in affinity for either F-actin or guanine nucleotide that occurs when the second ligand adds to the enzyme must be accompanied by an identical decrease in affinity for the complementary ligand. This energetically equivalent destabilization that occurs simultaneously at both ligand binding sites is the so-called interaction energy (51).

As shown in Fig. 3 (middle panel), the affinity of eEF1A for F-actin decreases 7.6-fold from 0.17 to 1.3 \mu M by the binding of GDP; therefore, from the above argument, the affinity of eEF1A for GDP also must decrease 7.6-fold from 2.5 to 19 \mu M by the binding of F-actin (Fig. 4A). Similarly, the affinity of eEF1A for F-actin decreases 7-fold by the binding of GTP (Fig. 3, lower panel); therefore, the affinity of eEF1A for GTP must decrease 7-fold from 0.6 to 4.2 \mu M by the binding of F-actin (Fig. 4B).

Thus, this thermodynamic model predicts a reciprocal reduction in the binding by eEF1A of both guanine nucleotide and F-actin when both ligands are present.

A Reduction in the \( K_m \) of GTP Hydrolysis Produced by F-actin Confirms the Thermodynamic Model—The above thermodynamic model makes the specific prediction that F-actin should reduce the affinity of eEF1A for GTP (Fig. 4B). To confirm this prediction directly, we employed a kinetic strategy utilizing the intrinsic GTPase activity of eEF1A. As shown in Fig. 2C, the kinetic Michaelis constant \( K_m \) for nucleotide hydrolysis is an excellent approximation of the thermodynamic affinity \( K_d \); therefore, in the presence of F-actin, a determination of \( K'_m \) also will provide the \( K'_d \) for GTP of the eEF1A-F-actin complex.

Initial rate studies were designed to determine the effect of F-actin on the \( K_m \) of GTP hydrolysis. The rates were determined under initial velocity conditions; the reactions were allowed to proceed to less than 10% of their end points. eEF1A (0.5 \mu M) was incubated with varying concentrations of GTP (0.4–48 \mu M) in the presence or absence of a saturating concentration of F-actin (18 \mu M, 15 \times K_d). The results presented in the form of a Lineweaver-Burke double reciprocal plot are shown in Fig. 5. The kinetic parameters \( K_m \) and \( k_{cat} \) for the reaction were obtained by fitting the data with hypero (35). In the absence of F-actin, the parameters are as follows: \( K_m = 0.71 \pm 0.05 \mu M \)

![Image](image-url)
The effect of F-actin on the kinetic constants of GTP hydrolysis by eEF1A. Varying concentrations of [γ-32P]GTP/GTP were incubated with nucleotide-free eEF1A (0.5 µM) in the presence (●) or absence (■) of saturating F-actin (15 µM). Following preincubation of eEF1A with F-actin, the reactions were initiated by the addition of nucleotide. Radiolabeled products were analyzed as in Fig. 2. Buffer conditions were as described in the legend to Fig. 1. Data are plotted as double reciprocal plots of initial velocity (V) versus GTP concentration.

and $k_{\text{cat}} = 1.35 \pm 0.03 \times 10^{-3}$ s$^{-1}$. In the presence of F-actin, the kinetic parameters increase to 2.96 ± 0.28 µM for $K_m$ and 2.16 ± 0.07 × 10$^{-3}$ s$^{-1}$ for $k_{\text{cat}}$. Given the separate errors generated by the different measurement techniques, the 4-fold increase in $K_m$ produced by F-actin is in relatively good agreement with the 7-fold increase in $K_d$ predicted by the thermodynamic model.

The indication of a stimulation of $k_{\text{cat}}$ by F-actin encouraged us to investigate further the effects of F-actin on the catalytic cycle of GTP hydrolysis by eEF1A (Fig. 6). Nucleotide-free eEF1A (1 µM) was equilibrated with a saturating concentration of F-actin (15 µM) at 22 °C. At $t = 0$ min, a mixture of GTP (30 µM containing 0.1% [γ-32P]GTP) was added, and aliquots of the reaction were removed and quenched at the indicated times.

Several conclusions can be drawn from the data presented in Fig. 6. First, F-actin clearly stimulates the rate of hydrolysis. The $k_{\text{cat}}$ in the presence of F-actin is 35% faster compared with the buffer alone control ($2.08 \times 10^{-3}$ s$^{-1}$ versus $1.55 \times 10^{-3}$ s$^{-1}$). Second, the binding of F-actin to eEF1A has no effect on the rate of product release although the rate of hydrolysis has increased. Under the conditions of the assay, an initial burst followed by a decline in product formation would occur if product release were rate-limiting. This is not the case; the reaction is linear in the presence and absence of F-actin over 3.5 enzyme turnovers. Third, the linearity of the reaction profile indicates that the rate of nucleotide exchange between bound GDP and free GTP is faster than the rate of hydrolysis ($2 \times 10^{-3}$ s$^{-1}$). If F-actin had affected the rate of exchange, the hydrolysis reaction would have progressively slowed due to the retarded regeneration of the GTP-eEF1A complex. Finally, the free accessibility of eEF1A internalized within an actin bundle to guanine nucleotide is further supported by the linearity of the early reaction. If the penetration of [γ-32P]GTP into an eEF1A:actin bundle were rate-limiting compared with the rate of catalysis, a lag in the initial production of 32P$_3$ compared with the eEF1A alone control would have been observed.

Estimation of the Interaction between eEF1A and F-actin in Vivo—To appreciate the physiological significance of the inter-

action between eEF1A and F-actin predicted by our thermodynamic analysis (Fig. 4), we wished to estimate in cells, using values of total cellular eEF1A and F-actin concentration and our measured binding constants, the degree of potential association between F-actin and the various nucleotide forms of eEF1A. The results of this estimation are summarized in Table III. Using data obtained from whole cell lysates of Dictyostelium (21) and two rat adenocarcinoma cell lines (16), the predominant form of eEF1A in the presence of 0.9 mM GTP and 0.1 mM GDP (64) is a complex of GTP-eEF1A:F-actin (75–90% of the total eEF1A).

**DISCUSSION**

Enzymatic Properties of eEF1A Alone—We have determined the reaction mechanism of eEF1A stabilized for optimal nucleotide binding. Our studies demonstrate that the combination of guanine nucleotide with glycerol is critical to stabilize fully the enzymatic properties of eEF1A. Loss of eEF1A activity is manifested by a reduction in the stoichiometry of nucleotide binding, increased protein-protein interactions and a reduction in the rate of GTP hydrolysis (data not shown). Due to the high sequence conservation between eEF1As, it is likely that all eEF1As require guanine nucleotide to stabilize their activity, a common property of many nucleotide-binding proteins (32, 53, 54). For other characterized eEF1As, the combination of lengthy nucleotide-free purification schemes with short half-lives for the GDP-eEF1A complex (19) suggests that even in the presence of glycerol, most eEF1As are isolated in a nucleotide-free state. Therefore, the possibility exists that previous studies describing eEF1A function may have utilized protein unstable toward nucleotide binding and with a predilection for aggregation (see Table I and Refs. 41–44).

The present study makes several observations about the catalytic cycle of GTP hydrolysis by eEF1A. The lack of product inhibition of GTP hydrolysis by eEF1A is in marked contrast to prokaryotic EF1A (EF-Tu), where GTP hydrolysis slows dramatically after one enzyme equivalent of product has formed (47). While information on differences in nucleotide affinity of eEF1A compared with prokaryotic EF1A (EF-Tu) has been available for many years (see Table I), this study is the first to
elucidate the relevance of this difference to the mechanism of GTP hydrolysis. For eEF1A, the relative difference in affinity between GDP and GTP alone is insufficient to predict a priori the rate-limiting step in the GTPase cycle. A direct comparison of the kinetic data for eEF1A and EF1A (EF-Tu) as pure GTPases shows that the reaction mechanisms are very different. Thus, while it is assumed that the general organization of eukaryotic protein synthesis resembles that of prokaryotic systems, certain molecular details of polypeptide elongation are clearly different.

The Effect of F-actin on the Enzymatic Properties of eEF1A—The intrinsic rate of GTP hydrolysis by eEF1A in vitro is too slow to account for the observed rates of elongation in vivo (18). Thus, eEF1A, like other GTPases, requires a stimulatory cofactor or GTPase-activating protein (15). For F-actin, we observe a 35% stimulation of the GTPase activity of eEF1A (Figs. 3 and 4). Based upon the proposed actin binding sites of eEF1A, it is likely that the N-terminal site in domain I is involved in this stimulation given its proximity to the effector loop proposed to mediate the effects of GTPase-activating proteins in other G proteins (15, 24). Yet the rate of hydrolysis in the presence of F-actin is still too slow to account for the in vivo rate of protein synthesis; therefore, it is doubtful that actin serves as a GTPase-activating protein for polypeptide elongation.

It is surprising that nucleotide exchange by eEF1A during the catalytic cycle is not grossly affected by the environment within an actin bundle. A dramatic change in the orientation of the N-terminal domain I with respect to the C-terminal domain III is presumed to accompany the transition from the GDP-bound to the GTP-bound state, in analogy to the prokaryotic homolog EF1A (EF-Tu) (49). One might anticipate a certain degree of steric hindrance to be imparted on a molecule tethered by the N and C termini between two actin filaments. Perhaps, a manifestation of this steric hindrance is the 7-fold reduced affinity of nucleotide binding noted above. Is it possible that the eEF1A:F-actin bundles “breathe” during nucleotide exchange as the cross-linking eEF1A’s “flex” from one nucleotide state to the other? Or perhaps nucleotide-dependent conformational changes are not displayed by eEF1A, consistent with the noted significant functional differences between prokaryotic and eukaryotic elongation factors.

The Binding of eEF1A to F-actin—It is unlikely that a change in the conformation of eEF1A induced by guanine nucleotides serves as a molecular switch to regulate the binding of eEF1A to F-actin, in that the affinities and stoichiometries of GDP:eEF1A and GTP:eEF1A for F-actin are equivalent (Table II). These similar affinities and stoichiometries suggest that if eEF1A exists in distinct nucleotide-dependent conformations, then these conformational states interact similarly with actin filaments. Yet the lack of a differential effect of GDP versus GTP on the affinity of eEF1A for F-actin does not rule out a nucleotide effect on the packing arrangement of actin filaments within an eEF1A:actin bundle (25).

For all nucleotide conditions (free, GDP, and GTP), there are approximately 1.5 eEF1As bound per actin monomer under saturating conditions (Table I) or, more realistically, three eEF1As per two actin monomers of an actin filament. There is no indication of oligomerization of the stabilized eEF1A used in these experiments as assessed by analytical gel filtration and chemical cross-linking (data not shown); therefore, this stoichiometry suggests that there are at least two eEF1A binding sites on an single actin monomer. Given the relative sizes of the actin and eEF1A molecules and the pitch of the actin filament helix, it is possible that when site 1 is saturated with eEF1A, site 2 can only bind another eEF1A every other actin monomer. The locations of these two potential eEF1A binding sites on the actin molecule are not known, although indirect evidence suggests that the N terminus of actin may be involved (26). Previous work indicates that the eEF1A molecule contains two actin binding sites located near the N and C termini (24), consistent with the ability of a single eEF1A molecule to cross-link actin filaments into bundles. Visualization of eEF1A cross-bridges in two-dimensional rafts of F-actin indicate an interfilament spacing of 120 Å, in agreement with the measured Stokes radius of a single globular 50,000-dalton polypeptide (20).

The Physiological Consequences of the Interaction between eEF1A and F-actin—eEF1A and actin are two of the most abundant proteins in eukaryotic cells. The observations that eEF1A and actin have a good binding affinity (Fig. 3) suggest that this interaction may be the most significant one for each molecule on a per mole basis. Our calculations in three different cell types indicate that 70–90% of total cell GTP:eEF1A is bound to actin (Table III).

An unusual characteristic of eEF1A is the ability to cross-link actin filaments into bundles whose properties are uniquely compared with other actin-containing structures: (i) the individual actin filaments within a bundle are ordered by eEF1A into a square-packed arrangement that excludes other actin cross-linking proteins (25); (ii) the rates of actin filament elongation, the amount of total polymer produced, and the half-lives of filaments are dependent on the relative concentration of eEF1A (23); and (iii) the association of eEF1A with actin in vivo is influenced by stimulation with extracellular ligands (16, 21, 27).

These properties of eEF1A:actin bundles suggest the estab-

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### TABLE III

| Type  | eEF1A<sub>total</sub> | F-actin<sub>Total</sub> | GDP · eEF1A<sub>Total</sub> | GTP · eEF1A<sub>Total</sub> | GDP · eEF1A · F-actin | GTP · eEF1A · F-actin |
|-------|----------------------|------------------------|-----------------------------|-----------------------------|------------------------|------------------------|
|       | μM                   | μM                     | Concentration               | Percentage<sup>a</sup>     | μM                     | Percentage<sup>a</sup> | μM                     | Percentage<sup>a</sup> | μM                     | Percentage<sup>a</sup> | μM                     | Percentage<sup>a</sup> |
| Dicty | 75<sup>b</sup>        | 89<sup>b</sup>         | 0.04                        | 0.05                        | 1.5                    | 2                      | 7                      | 9                      | 66                     | 88                     |
| MTLn3 | 57<sup>b</sup>        | 76<sup>b</sup>         | 0.02                        | 0.03                        | 0.9                   | 1.6                    | 5                      | 9                      | 51                     | 89                     |
| MTC   | 37<sup>b</sup>        | 31<sup>b</sup>         | 0.2                         | 0.5                         | 7                     | 19                     | 3                      | 8                      | 27                     | 73                     |

<sup>a</sup> Dicty, D. discoideum strain AX<sub>3</sub>; MTLn3 and MTC are metastatic and nonmetastatic, respectively, cell lines derived from rat adenocarcinoma (see Ref. 16).

<sup>b</sup> Percentage of total cellular eEF1A.

<sup>c</sup> From Ref. 21.

<sup>d</sup> From Ref. 56.

<sup>e</sup> From Ref. 16.

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lishment of an unique actin microcompartment within cytoplasm that may correlate with changes in cell motility and mRNA translation. Recently, the prediction of competition between aminoacyl-tRNA and actin for binding to eEF1A (55) was confirmed experimentally, and it suggests that the binding sites for each ligand overlap (24). This competition may indicate that F-actin can sequester eEF1A away from aminoacyl-tRNA, thereby reorienting the eEF1A bound to F-actin incompetent to participate in translation. However, in response to cell stimulation, the eEF1A could release from actin, bind with aminoacyl-tRNA, and then interact with neighboring translational co-factors also bound to actin and provide a burst of localized protein synthesis (22). This eEF1A sequestration-release cycle may provide a mechanism for cells to spatially and temporally regulate the synthesis of specific proteins to specific intracellular compartments in response to extracellular stimulation. In addition, actin may sequester eEF1A away from other potential ligands, thereby providing a regulatory mechanism for other eEF1A-mediated cellular processes as well.

The validity of the actin-eEF1A sequestration model hinges upon the relative affinities of eEF1A for actin and its other ligands, and the relative concentration of these species within specific intracellular compartments. The further determination of binding constants and localized concentrations for other ligands of eEF1A will help to refine our thermodynamic model of binding constants and localized concentrations for other specific intracellular compartments. The further determination of binding constants and localized concentrations for other ligands of eEF1A will help to refine our thermodynamic model and our understanding of the mechanisms controlling localized processes involving eEF1A in cells.

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