Most G-proteins require a guanine nucleotide exchange factor (GEF) to regulate a variety of critical cellular processes. Interestingly, a small number of G-proteins switch between the active and inactive forms without a GEF. Translation Elongation Factor 1A (eEF1A) normally requires the GEF eEF1Bα to accelerate nucleotide dissociation. However, several mutant forms of eEF1A are functional independent of this essential regulator in vivo. GEF-independent eEF1A mutations localize close to the G-protein motifs that are crucial for nucleotide binding. Kinetic analysis demonstrated that reduced GDP affinity correlates with wild type growth and high translation activities of GEF-independent mutants. Furthermore, the mutant forms show an 11 to 22-fold increase in rates of GDP dissociation from eEF1A compared to the wild type protein. All mutant forms have dramatically enhanced stability at elevated temperatures. This, coupled with data demonstrating that eEF1A is also more stable in the presence of nucleotides, suggests that both the GEF and nucleotide have stabilizing effects on eEF1A. The biochemical properties of these eEF1A mutants provide insight into the mechanism behind GEF-independent G-protein function.

GTPases regulate a variety of cellular functions with a conserved mechanism of nucleotide binding and hydrolysis. Signal transduction, control of cell cycle and differentiation during cell division, protein biosynthesis, vesicular trafficking and translocation of membrane proteins are key cellular processes where GTPases play critical roles. Based on their functional roles, domain structures or sizes, the superfamily of GTPases can be divided into many families including small G proteins (Ras GTPase superfamily), heterotrimeric G proteins, and the translation factor family (1). All GTPases share a G-domain with conserved sequence elements such as switch I and II, the P-loop (phosphate binding loop) and the NKXD element (2). Guanine nucleotides form specific interactions with these sites, which are modulated by G-protein accessory factors to create the switch mechanism between the active and inactive forms (1,3).

GTPase activation factors (GAPs) stimulate GTP hydrolysis resulting in the inactive GDP-bound form. Guanine nucleotide exchange factors (GEFs) catalyze GDP release by reducing the nucleotide affinity (1). This allows G-proteins to rebind GTP due to a higher cellular concentration of GTP and thus switch to their active form. The GEFs interact with the switch I and II regions while inserting residues close to or into the P-loop and Mg+2 binding site. The insertion of the GEF residues perturbs the interaction surface in the phosphate binding region resulting in the release of phosphate groups which in turn cause dissociation of the nucleotide. In contrast to the mechanism of exchange and the G-proteins themselves, GEFs show little to no conservation in sequence or structure (3).

The crystal structures of the majority of G-proteins solved in the presence of nucleotides show that the β-phosphate of the nucleotide interacts with the P-loop and this interaction is considered to be the most important element for the tight binding of nucleotide (1). Another important contributor to binding affinity and specificity is the NKXD element, which interacts with the nucleotide base (2). Until recently, the order of events that leads to nucleotide release and the region of the nucleotide released first were unclear. However,
a sequence of interactions was proposed at least for some G-proteins. According to the model, upon binding of the GEF to the G-protein, the phosphate groups are released first, then the base of the entering nucleotide binds to the NKXD motif and displaces the GEF (4).

While most of the GTPases require a GEF, some G-proteins can function efficiently without an exchange factor. Typically, GEFs accelerate the nucleotide release from G-proteins, which is normally a slow process. A G-protein without a GEF likely allows nucleotide release rapid enough for cell survival. G-proteins could maintain rapid nucleotide release rates that lead to GEF independence in several ways. Lower affinity of GDP to the G-protein could allow a G-protein to function without a GEF. In addition, the G-protein may not require the separate level of regulation of activity typically performed by the GEF. The eukaryotic proteins translation Elongation Factor 2 (eEF2), Release Factor 3 (eRF3), Initiation Factor 5B (eIF5B), and the GEFs Hbs1p and Guf1p apparently function independently of a GEF. This is mostly explained by reduced GDP affinity or higher dissociation rate constants for GDP release from the G-protein in the absence of GEF. For example, the rate constants for GDP dissociation from both eIF5B and SelB (bacterial EFSec) are higher than other translation factors (5,6) and EFSec has lower affinity for GDP (7,8). This, coupled with the higher level of GTP over GDP in the cell, allows spontaneous regeneration of the active form of the protein (6,8,9). Some studies suggest that the ribosome acts as a GEF for prokaryotic RF-3 and EF-G (10). The variety for the GEF requirement and the sequence and structural diversity of GEFs implies that GEF proteins might have gained different functions which may be specific to the GTPase:GEF complex, cell type or the organism.

One of the G-proteins in the translation family is Eukaryotic Translation Elongation Factor (eEF1A). It binds and recruits aminoacyl-tRNAs (aa-tRNAs) to the A-site of the ribosome. eEF1A is part of the eEF1 complex including the eEF1B subunits. eEF1B is composed of the α and γ subunits in fungi and a third β subunit is present in metazoans. The eEF1Bα subunit performs the catalytic GEF function for eEF1A. The γ subunit is likely a regulatory subunit, since the eEF1Bγ complex has a proposed role in the oxidative stress response pathway (11). The crystal structure of *Saccharomyces cerevisiae* eEF1A with the catalytic C-terminus of eEF1Bα shows that one face of eEF1Bα interacts with domain I, while the other interacts with domain II (12). Domain I contains the nucleotide and Mg^{2+} binding site, while domain II is the proposed aa-tRNA binding site of eEF1A. In addition to its established role as a GEF for eEF1A and accelerating the rate of GDP dissociation by 700-fold (13), eEF1Bα also affects translational fidelity (14). Based on the crystal structures of bacterial and eukaryotic EF-Tu/eEF1As, mammalian eEF1A has evolved from bacterial EF-Tu by the insertion of about 70 amino acids into the loop regions between the domains (9). *S. cerevisiae* eEF1A has 81% identity and 89% similarity to human eEF1A (15,16). These results suggest that while the structure and the function of eEF1A are well conserved, the GEF for eEF1A has gained more complexity and perhaps more functions throughout evolution.

In *S. cerevisiae*, the TEF5 gene encoding eEF1Bα is essential *in vivo* (17). Interestingly, the requirement for the TEF5 gene can be suppressed by the presence of excess substrate, eEF1A. Such an eEF1Bα-deficient strain, however, shows defects in growth and translation (18). Two independent, unbiased genetic screens performed to isolate suppressors of the eEF1Bα requirement *in vivo* yielded only eEF1A mutations. The mutant forms of eEF1A that function as suppressors of the eEF1Bα deficiency allow growth similar to a wild type eEF1A strain. In order to analyze the effect of suppressor mutations independent of any other eEF1 components, strains lacking eEF1Bα and both chromosomal eEF1A genes and thus expressing only the mutant form of eEF1A genes were prepared. Surprisingly, these strains show no growth defects and little to no reduction in total translation (19). Interestingly, all mutations map to the nucleotide-binding domain of eEF1A. Each mutation is in very close proximity to at least one of the conserved sequence elements of the G-protein, which suggest nucleotide affinity to eEF1A might be affected creating an open
conformation to allow accelerated GDP release without eEF1Bα.

In order to determine the mechanism of the bypass suppression of an essential GEF, we analyzed the effect of mutant forms of eEF1A on nucleotide binding by fluorescence resonance energy transfer (FRET) from hydrophobic residues of eEF1A to fluorescently labeled (mant) nucleotides. The equilibrium dissociation constants (K_d) for mant-GDP binding to eEF1A mutant forms increased up to 37-fold compared to that of wild type eEF1A, indicating reduced GDP affinity. Using stopped-flow kinetics, the mutant forms of eEF1A displayed increased GDP dissociation rates up to 22-fold compared to the wild type protein. However, the K_d values for mantGMPPNP, a nonhydrolyzable homolog of GTP, were essentially unchanged indicating that the selective pressure reduces GDP, but not GTP binding. Although the mutations do not cause a fundamental change in the native state of the protein as observed by Circular Dichroism (CD) Spectroscopy, the mutant forms showed dramatically increased stability. Enhanced stability was also observed when eEF1A was bound to guanine nucleotides. Thus, this study demonstrates that the GEF eEF1Bα as well as the distribution of the nucleotide-bound state of eEF1A within the cell likely contribute to stabilizing the protein. The consequences of these eEF1A mutations on the specificity of effects on GDP versus GTP binding raises the questions of evolutionary development of GEF function and independence as well as G-protein complexity.

**Experimental Procedures**

_Yeast techniques and mutant preparation: S. cerevisiae_ strains used in this study are listed in Table 1. _Escherichia coli_ DH5α was used for plasmid preparation. Standard yeast genetic methods were employed (20). Yeast cells were grown in YEPD (1% Bacto yeast extract, 2% peptone, 2% dextrose) as the carbon source (21). The R164K, T22S, A112T and A117V eEF1A mutants were prepared in pTKB754 (_TEF1_ URA3) by PCR mutagenesis using the Quikchange method (Stratagene). The resulting plasmids were transformed into MC213 (_TEF2 TRP1_), loss of wild-type eEF1A plasmid was monitored by growth on 5-fluoroanthranilic acid (5-FAA) (22), and the recovered strains TKY961, TKY963, TKY964 and TKY965, respectively were used for the purification of the mutant proteins.

**Protein purification:** eEF1A was purified as described (13,23) from strains TKY368, TKY961, TKY963, TKY964 and TKY965 (Table 1) with the following modifications. The eluted and dialyzed material from the protein solution was applied to CM-52 cation exchanger, pre-equilibrated with buffer 1 (20 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 25% glycerol, 1 mM DTT, 0.2 mM PMSF and 1 µg/ml Aprotinin). The CM-52 column was washed with buffer 1 with 50 mM KCl and eluted using a 50 mM to 300 mM KCl salt gradient of buffer 1. eEF1A containing fractions were dialyzed overnight against 10 volumes of buffer 1 with 100 mM KCl and stored in aliquots at -80°C. Protein fractions used for assays were >95% pure as determined by SDS-PAGE.

2’-(or 3’)-O-N-methylanthraniloyl (mant) labeled guanine nucleotide binding assay: mant-GMPPNP and mant-GDP were both purchased from Molecular Probes and purified with 1-ml Hi-Trap DEAE-Sepharose Fast Flow column (Amersham Biosciences) using an AKTA fast-protein liquid chromatography system (Amersham Biosciences). Nucleotide-containing fractions were combined, lyophilized using a speed vacuum, resuspended in 100 µl of dH2O, and stored at -20 °C (13).

The binding affinity for mant-GDP or mantGMMPNP to wild type or the mutant forms of eEF1A was measured by a fluorimetric titration assay using a FluoroMax-3 spectrofluorimeter (Horiba Jobin Yvon Inc.). All assays were performed at 25 °C. Wild type or mutant forms of eEF1A (1 µM) in 2.5 ml of binding buffer (10% glycerol, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM MgCl2) were placed in a 10- x 10- x 40-mm quartz cuvette with a magnetic stirring bar. Increasing concentrations of mant-nucleotide were added with continuous stirring for three min. Fluorescence changes of the mant-nucleotides (F_0obs) were monitored upon indirect excitation via fluorescence resonance energy transfer (FRET). FRET excited tryptophans or tyrosines
of eEF1A at a wavelength of 280 nm and the emission wavelength of 440 nm was for the mant moiety of the nucleotides. The slit widths were 1.05 nm for both wavelengths. The protein and nucleotide complex-dependent fluorescence values \( (F_{em}) \) were obtained by correcting for titration volume and inner filter effect using the equation \( F_{em} = \frac{F_{obs} \times (V_f/V_o)}{10^{0.5 (A_{em(280)} + A_{em(440)})^2}} \), plotted against mant-GMPPNP or mant-GDP concentrations and fit to the equations \( F_{em} = C + f_{Eb} M + f_{Mt} M_t \) where \( V_f \) is the final volume, \( V_o \) is the initial volume, \( A_{ex(280)} \) is the excitation absorbance of mant-nucleotide, and \( A_{em(440)} \) is the emission absorbance of mant-nucleotide, \( C \) is background fluorescence, \( f_{Mt} \) is the fluorescence coefficient of free mant-nucleotide, \( f_{Eb} \) is the fluorescence coefficient of nucleotide-bound to eEF1A, \( E_t \) is the total eEF1A protein concentration, \( M_t \) is the total concentration of mant-nucleotide, the concentration of eEF1A bound to mant-nucleotide is \( E_b \), and \( K_d \) is the mant-GMPPNP or mant-GDP dissociation constant.

**Fluorescence Stopped-Flow Kinetic Experiments:** Stopped-flow experiments using mant-GDP (Molecular probes) were done in SF-2001 (KinTek Corp) stopped-flow spectrophotometer equipped with a photomultiplier detection system as described in (13).

**Circular Dichroism (CD) measurements:** CD measurements were made using Circular Dichroism Spectrometer Model 400 (Aviv Biomedical Inc.). The protein concentrations of the purified fractions were determined from the measurement of their different spectra in 6 M guanidine-HCl between pH 12.5 and pH 6.0. The molar concentration (MC) of protein in the cuvette was calculated using the formula \( MC = A/(2.357Y+830W) \), where \( A \) is the absorbance at 294 nm, \( Y \) is the number of tyrosines and \( W \) is the number of tryptophans. The stock solutions were diluted to 0.2-0.4 mg/ml in purification buffer 1 (20 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 25% glycerol, 1 mM DTT, 0.2 mM PMSF and 1 µg/ml Aprotinin) to a total volume of 300 µl in cells with a 0.1 cm path wavelength. The wavelength was set to 200-260 nm with 0.5 nm intervals. The wavelength measurements were carried out at 25°C. After the temperature scan at 222 nm to obtain the melting curve, the wavelength scan was measured at 70°C and 25°C again after the samples were cooled down to test the refolding properties of the proteins. The CD spectra were analyzed using neural network analysis programs to determine \( \alpha \)-helical, antiparallel and parallel \( \beta \)-structure, turns and the remainder content of the proteins (24,25). The Aviv Macro Editor program was used to collect the wavelength spectra as a function of temperature. The cells were equilibrated for 2 minutes at 5°C integrals and the spectra were accumulated three times at a given temperature. The convex constraint algorithm (CCA) was used to deconvolute the spectra to obtain the minimum number of basis spectra needed to fit the data (26,27). All CD data were reported in deg cm\(^2\) dmol\(^{-1}\) ([θ]).

**RESULTS**

*Interactions of guanine nucleotides with eEF1A are well represented by the change in mant-nucleotide fluorescence.* In order to determine the baseline guanine nucleotide binding characteristics of eEF1A, FRET from tryptophans and tyrosines of eEF1A to the mant fluorophore of GDP or GMPPNP was determined. Enhanced mant-nucleotide fluorescence with eEF1A (Figures 1B and 2B) compared with mant-nucleotides alone (Figures 1A and 2A) is the indication of FRET between eEF1A and the mant fluorophore of GDP or GMPPNP was determined. Enhanced mant-nucleotide fluorescence with eEF1A (Figures 1B and 2B) compared with mant-nucleotides alone (Figures 1A and 2A) is the indication of FRET between eEF1A and the mant-nucleotides. In order to determine guanine nucleotide binding affinity of eEF1A, \( K_d \) values of eEF1A for mantGDP and mantGMPPNP were determined by a fluorimetric titration assay. The \( K_d \) for mantGDP binding to eEF1A was determined as 0.095 µM (Figure 1B). This value is in agreement with a \( K_d \) of 1 µM for unlabeled GDP and eEF1A (28) and our previously published \( K_d \) of 0.18 µM for mantGDP binding to eEF1A determined by stopped flow kinetics (13). To determine GTP binding affinity of eEF1A, the mant-labeled non-hydrolysable analog of GTP, mantGMPPNP, was used. The previously published \( K_d \) of the eEF1A:GTP complex is 0.7 µM (28). The \( K_d \) for mantGMPPNP binding to eEF1A was measured as 0.52 µM (Figure 2B) indicating that mantGMPPNP binding to eEF1A
is a good representation of unmodified GTP binding to eEF1A.

**Mutations of eEF1A that suppress the requirement for eEF1Bα reduce GDP affinity up to 37-fold.** All of the mutations that allow eEF1A to function without its guanine nucleotide exchange factor eEF1Bα cluster in GTP-binding domain (19). In order to determine if these mutations affect nucleotide affinity in the absence of the exchange factor, we analyzed the GDP binding to the mutant forms of eEF1A by the mantGDP fluorescence assay. Upon binding of the nucleotide to the eEF1A mutants, an increase in fluorescence was observed and the data were fitted to determine $K_d$ values for each mutant. The values obtained were 3.54 (T22S), 2.66 (R164K), 1.80 (A112T) and 1.18 μM (A117V) (Figure 1C-F, Table 2), which represent a 12 to 37-fold increase in $K_d$ as compared to wild type eEF1A (0.095 μM, Figure 1B). Thus, one reason for the suppression of the requirement for the guanine nucleotide exchange factor, eEF1Bα, is the reduced affinity of the mutant forms to GDP compared to the wild type eEF1A.

**GEF-independent mutations of eEF1A do not affect GTP binding.** eEF1Bα catalyzes the release of GDP to allow the inactive (GDP-bound) form of eEF1A to recycle back to its active (GTP-bound) form. In order to determine whether these eEF1A mutations reduce binding of both GDP and GTP or specifically GDP affinity, the $K_d$ of the eEF1A mutant proteins for mantGMPPNP were obtained. The change in fluorescence values was plotted against the increasing concentration of mantGMPPNP and fitted to the equations to give $K_d$ values of 1.01 (A112T), 1.09 (A117V), 0.47 (T22S) and 0.41 μM (R164K) (Figure 2C-F, Table 2). The fact that the $K_d$ values of the mutant forms are very similar to the $K_d$ value of wild type eEF1A (0.52 μM, Figure 2B) indicates that genetic selection during the isolation of the suppressors for the requirement of eEF1Bα targets GDP but not GTP affinity.

**GEF-independent mutants of eEF1A dissociate GDP at a higher rate.** To determine if the mutations cause rapid spontaneous GDP dissociation from eEF1A in the absence of its GEF, we measured the dissociation rate constant of the mant-GDP:eEF1A complex using stopped-flow kinetics. Mutant forms of eEF1A were prebound to mantGDP and then rapidly mixed with an excess of nonfluorescent GDP. The rate of mant nucleotide release was obtained by monitoring the decrease in fluorescence over time. The fluorescent intensity decayed exponentially on the order of seconds. The time course of mantGDP displacement from the binary complex of each mutant by excess GDP was fitted by a single-exponential term, yielding dissociation rate constants ($k_{off}$) of 3.89 (A117V), 2.54 (R164K), 1.96 (A112T) and 1.88 (T22S) s$^{-1}$ (Figure 3A-D, Table 2). The average dissociation rate constant of wild type eEF1A is 0.17 s$^{-1}$ (13). These data show that the nucleotide dissociation rate constants were stimulated 11 to 22-fold in the GEF-independent forms of eEF1A. These results support the contributions of higher GDP release rates in addition to reduced nucleotide affinity in the GEF independence phenotype of a G-protein.

**GEF-independent forms of eEF1A increase the thermostability of the protein.** In order to determine if these mutations affect the secondary structure of eEF1A, Circular Dichroism (CD) spectroscopy was performed with wild type and mutant forms of eEF1A. eEF1A is α-helical in the GTP binding domain (domain I), while domains II and III are composed of antiparallel β sheets. CD values at 25°C plotted against the wavelength to observe the formation of bands for α-helices and β-sheets. There was no difference between the wild type (●, Figure 4A) and the eEF1A mutant forms such as A112T (▲, Figure 4A), which suggest that they all have similar secondary structure.

In addition to providing information on the secondary structural composition of the proteins, CD spectra also allow analysis of unfolding and folding properties of a protein as a function of temperature (29,30). In order to investigate the thermodynamics of protein folding, the ellipticity was monitored at a single wavelength (222 nm). The resulting melting curves of wild type and the mutant proteins indicated that the mutations caused a different unfolding pattern compared to wild type eEF1A. Intriguingly, the wild type eEF1A completely unfolds at 70°C (○) whereas the A112T form of eEF1A retained secondary structure (▲, Figure 4A).
4A). Other mutant forms of eEF1A, R146K (○), T22S (▲), A112T (▲) and A117V (■), also increase protein thermostability in comparison to wild type (●) at 70°C (Figure 4B).

By using neural networks, CD spectra were further analyzed to estimate α-helical, antiparallel and parallel β-structure, turns and the remainder content of the proteins. In each mutant, the estimates of the fraction of each secondary structure in native state were similar to the wild type. However, in denatured states, while the α-helical content of wild type protein reduced to 14% and total β content increased to 35%, the total helix of the mutant forms were up to 20% and the total β content remained between 25-29% (Table 3)

To determine true thermodynamic properties by CD or other spectroscopic techniques, unfolding and the folding of a protein should be reversible. After melting, the eEF1A samples were cooled down to 25°C and CD spectra at 25°C showed that unfolding of eEF1A is irreversible. Thus complete CD spectra as a function of temperature were collected to determine the relative stability by comparing the unfolding intermediates of the wild type and the mutant forms (27). To determine unfolding transitions, a macro writing program was run from 25°C to 75°C with 5°C intervals and the wavelength scan was obtained at a range of 200-260 nm at each temperature. To analyze the change in the spectrum of the proteins as a function of temperature, the CD spectra at different temperatures were deconvoluted by the CCA. The minimum number of basis spectra to fit the data was determined as three, which corresponds to the number of transition states of unfolding. The graphs obtained after deconvolution of the spectra showed that the first intermediates of the unfolding (■) are similar between the wild type (Figure 5A) and the mutant proteins (Figure 5B-E). The second intermediates (▲), however, show that the wild type protein has a distinct peak below 210 nm (arrow), corresponding to more β-sheet characteristics not seen in the mutants. While the third state (●) of wild type eEF1A shows a completely unstructured protein (Figure 5A), some of the secondary structure was protected at the third state of the mutant proteins in correlation with the percent compositions of the secondary structures (Figure 5B (A112T), 5C (R164K), 5D (A117V) and 5E (T22S)). The fractions of each curve indicate that at around 40-50°C, both mutant and wild type proteins leave the first state of the transition and move to the second state. Between 50-55°C, the third states of the proteins emerge.

The unfolding of a protein usually starts with α-helical segments turning into β-sheets. The higher β-sheet content of the second intermediate of wild type eEF1A indicates that the wild type protein starts to denature earlier than most of the mutant proteins. Interestingly, the deeper curve and higher negative values of ellipticity of the wild type protein moving to the third state of the transition as the unfolded state, differs from the mutant proteins. The mutant proteins have a third intermediate, which corresponds to a state between the second and third states of wild type eEF1A. A112T and R164K forms of the proteins also delay the transitions by 2-5 °C as compared to the wild type protein, corresponding to the higher values of midpoint of the unfolding transitions (Tm).

Nucleotides stabilize eEF1A. EF-Tu, the bacterial homolog of eEF1A is reported to be more stable in the presence of the nucleotides (31). As the mutations reduce nucleotide affinity but increase the stability of eEF1A, we measured CD spectra of eEF1A in the presence of GDP and GMPPNP to determine the effect of nucleotides on eEF1A stability. The measured Kd value for GTP:eEF1A is 1μM and that of GMPPNP:eEF1A is 0.7 μM in the presence of 1 μM eEF1A. Therefore, a 5-fold excess of nucleotides were incubated with purified eEF1A to ensure binding. Spectra collected as a function of temperature showed that the first intermediates of the unfolding (■) of apo eEF1A (Figure 6A) or for eEF1A in the presence of GDP (Figure 6B) or GMPPNP (Figure 6C) are similar with high negative ellipticity values. However, as the proteins move to the second intermediate (▲), the apo protein loses the peaks at specific wavelengths. Both nucleotide bound forms show a distinct peak close to 210 nm indicated by arrows. This peak likely indicates the presence of secondary structure, mostly β-sheets. At the third state of transition (●), both the apo and GDP bound forms of the eEF1A have lost the secondary structure, but the
GMPPNP bound form still shows the negative peaks. The fractions of each curve for eEF1A in the presence of nucleotide are similar to the mutant forms (data not shown). These results indicate that eEF1A is more stable when it is bound to the nucleotides and the GTP-bound form of the eEF1A is the most stable form of all three.

DISCUSSION

Most G-proteins have accessory proteins which help to regulate their activity via the classic “molecular switch”. However, some G-proteins can perform nucleotide exchange with no requirement for a GEF. In addition, while G-proteins form well conserved families and hydrolyze GTP by similar mechanisms, GEFs form a diverse group of molecules (3). To investigate the underlying causes for the GEF requirement of different G-proteins, we analyzed a GEF-independent G-protein system created based on an unbiased genetic screen. Data reported here indicate that genetic selection targets nucleotide affinity and release rates, which is consistent with studies showing that the binding affinity for the nucleotides and the association/dissociation rates account for naturally occurring GEF-independence (5,6).

The assays performed with fluorescently labeled nucleotides to assay eEF1A:GTP and eEF1A:GDP binding demonstrate that these nucleotides are a good representation of nucleotide binding. The mutant proteins have up to 37-fold lower affinity for GDP compared to the wild type protein with little effect on the GMPPNP affinity. Examination of the structure of the eEF1A-eEF1Bα complex in the presence of nucleotides explains the lower affinity of the mutations. The altered residues most likely change the electrostatic interactions of the phosphate groups and/or the guanine base of GDP to the conserved elements of the G-domain of eEF1A.

The interactions of the amino acid residues and the putative effect of the mutations on the structure of eEF1A were evaluated using PyMol program (32) and DeepView/ Swiss-Pdb viewer (33). According to these analyses, T22 forms a hydrogen bond with the α-phosphate of GDP (Figure 7A). In the T22S mutant, disruption of this interaction is predicted to cause GDP to dissociate easily. The T22S mutant has the largest Kd, correlating with its critical position in the structure of eEF1A. GDP binding is most reduced in this mutant and the GDP dissociation rate is the lowest.

R164 is as close as 5.7 Angstrom (Å) to the NKXD element of eEF1A (Figure 7B) suggesting that it may play an important role in increasing the affinity of the guanine base. The Kd for GDP in the R164K mutant is reduced less than T22S, but the koff is increased about 33% compared to T22S.

The A117 and A112 residues are located at the upper and lower tip of the same β-strand, which is between a β-strand that connects to the P-loop and a second β-strand that connects to the NKXD element (Figure 7B). This could explain why mutations A112T and A117V have similar Kd values for GDP (Table 2). A117 is located between the I116 and G118 residues that interact with NKXD via hydrogen bonds and A117 itself is only 4.3 Å away from the NKXD element. Since the NKXD motif stabilizes the guanine base, a disruption of these interactions may cause an open conformation aiding in the release of GDP. This is most pronounced for the A117V mutation, which has the highest koff for GDP. The distance between A117 and R164 is the shortest among all pairs of mutations. The distance between A112 and the P loop is 8.9 Å, but I13, the first residue before the P loop, is only 5 Å away from the A112. Therefore, A112T could also allow the P-loop to move and facilitate GDP release without eEF1Bα insertion (Figure 7A). The consequences of the disruption of these critical interactions point out the crucial role of the P-loop and the NKXD element for the exchange mechanism.

The mutant eEF1A:nucleotide binding data indicate that the genetic selection for GEF independence targets GDP affinity and GTP affinity remains unaltered. This result correlates with the effect of eEF1Bα on the nucleotide affinity of eEF1A. eEF1Bα catalyzes the exchange reaction by increasing the rate of GDP dissociation up to 700 fold (13). However, this effect is only related to GDP, but not GTP affinity (unpublished data). This data shows that the GEF independent mutants mimic the wild
type exchange factor complex at least for the interaction with the guanine nucleotides. While the mutations increase the rate of dissociation up to 22-fold compared to wild type eEF1A, it seems that the combination of both reduced affinity and the higher rates of nucleotide dissociation result in the GEF independent phenotype. For R164K, A112T and A117V mutant forms, lower dissociation rates are compensated for by a greater reduction in GDP binding. Although the reduced binding affinity has been suggested to be the reason for the higher dissociation rates, they might be two independent but coordinated events compensating each other to keep the exchange rate above a certain threshold for viability. Interestingly, T22S and A112T forms of eEF1A, which showed the smallest increase in the \( k_{\text{off}} \) compared to the other two mutants, also have reduced translation efficiency (19). This finding supports that reduced exchange activity usually correlates with lower translation rates. Overall, the mutations modulate the electrostatic interactions such that eEF1A, on its own, allows the exchange reaction to occur at a sufficient rate for the maintenance of cellular functions. This provides functional information on how cellular systems are dynamically regulated and readily adapt to the limiting conditions.

Most G-proteins are reported to be unstable without bound nucleotides and exchange factors (31,34,35). This is likely because most enzymes are more stable and resistant to the proteolysis in the presence of their substrates. The effect of the GEF independent mutants and guanine nucleotides on the structure of eEF1A was investigated by testing their ability to affect the stability of eEF1A at elevated temperatures. The CD spectra of the mutant and wild type proteins show that GEF independent forms of eEF1A stabilize a normally unstable protein. Despite many efforts, the crystal structure of the eEF1A without any factor bound has not been solved. This may be a further indication that eEF1B\(\alpha\) stabilizes eEF1A. The eEF1A-GTP complex was more stable than the eEF1A-GDP complex. This data is consistent with some G-proteins being more thermostable in the GTP-bound conformation than in the GDP-bound conformation, which may be explained by the extra interaction with the \( \gamma \)-phosphate residue of GTP (36,37). The structures of GTP- and GDP-bound ET-Tu show that a large rearrangement occurs upon GTP hydrolysis (36,38). In addition, the structure of eEF1A:eEF1B\(\alpha\) complex resembles GTP-bound EF-Tu (12,39). A similar closed nature of GTP-bound eEF1A might also account for the greater thermostability of eEF1A in the presence of GTP and the higher stability of the mutant forms of eEF1A in the absence of eEF1B\(\alpha\). Thus, in addition to modulating nucleotide affinity, GEF independent forms also increase the stability to perhaps compensate for another role of eEF1B\(\alpha\). This finding also sheds light on the isolation and designation of the historical bacterial T fraction (the EF-Tu.EF-Ts complex) as two components: one stable component, T\(\alpha\), and a heat-labile, unstable component T\(\alpha\), the bacterial homolog of eEF1A (40).

In addition, thermostability of the mutant forms of eEF1A correlates with the initial conditions of the screens which yielded the suppressors of eEF1B\(\alpha\) deficiency. The eEF1B\(\alpha\) deficient strain used for the EMS mutagenesis was thermosensitive and cold sensitive. In the first screen, the colonies were screened for growth at 37°C. The resulting mutations all suppressed the temperature sensitive phenotype of the strain overexpressing eEF1A and lacking eEF1B\(\alpha\) but not the cold sensitivity. To avoid this bias the screen was repeated looking at growth at 24°C and 37°C, and again a significant overlapping group of mutants was obtained compared to the first screen and all grew better at 37°C. In fact, suppression was clearly more pronounced at 37°C (19). Therefore, in this paper, we showed that the \textit{in vitro} characteristics of the proteins are consistent with the \textit{in vivo} phenotype of the strains from the original screen.

In some cases the affinity of a G-protein for GDP is much higher than the GTP so the requirement for an exchange factor is obvious. However, the weaker binding of GDP compared to GTP and high dissociation rate constants are an indication for a G-protein without exchange factor dependence such as for eIF5B and bacterial eEFSec (5,6). Sequence alignments were analyzed to investigate the potential significance of the differences and the similarities between the GEF independent G-
proteins (eEF2, eIF5B, eRF3, eEFSec, Guf1p and Hbs1p) and GEF-independent forms of eEF1A. T22 and A112 are both 83% identical between GEF-independent proteins from yeast and 77% and 66% identical between human GEF-independent proteins respectively. The same residues are 100% conserved in eEF1A/EF-Tu from 11 selected organisms from bacteria to human. The A117 and R164K residues are 85% identical in eEF1A/EF-Tu, and the same residues in human and yeast proteins functioning without a GEF show only a 25-35% identity. Interestingly, other than the K substitute for R164 in mitochondrial IF-2 (mIF-2), none of the mutations that allow eEF1A to be GEF independent occur in other GEF independent proteins (Figure 7C). Thus, there appears to be selective pressure to maintain these residues in the elongation factors. This indicates that the mechanisms that result in altered nucleotide exchange activities between eEF1A and other GEF independent G-proteins are not same, but that eEF1A can also act as a GEF-independent G-protein by remodeling its nucleotide-G-protein interactions at highly conserved sites.

ACKNOWLEDGEMENTS

We thank the members of the Kinzy lab for helpful comments. Thanks to Yvette Pittman for help with kinetic studies, Dr. Norma Greenfield for her assistance on obtaining and analyzing CD spectra, Dr. Smita Patel for use of stopped-flow equipment and Peter Krueger for the sequence analysis of GEF independent G-proteins. This work was supported by NIH GM 57483.

REFERENCES

1. Vetter, I. R., and Wittinghofer, A. (2001) Science 294, 1299-1304
2. Dever, T. E., Glynias, M. J., and Merrick, W. C. (1987) Proc. Natl. Acad. Sci. USA 84, 1814-1818
3. Sprang, S. R., and Coleman, D. E. (1998) Cell 95, 155-158
4. Bos, J. L., Rehmann, H., and Wittinghofer, A. (2007) Cell 129, 865-877
5. Pisareva, V. P., Hellen, C. U., and Pestova, T. V. (2007) Biochemistry 46(10), 2622-2629
6. Thanbichler, M., Bock, A., and Goody, R. S. (2000) J Biol Chem 275(27), 20458-20466
7. Tujebajeva, R. M., Copeland, P. R., Xu, X. M., Carlson, B. A., Harney, J. W., Driscoll, D. M., Hatfield, D. L., and Berry, M. J. (2000) EMBO Rep 1(2), 158-163
8. Fangegaltier, D., Hubert, N., Yamada, T., Mizutani, K., Carbon, P., and Krol, A. (2000) Embo J 19(17), 4796-4805
9. Merrick, W. C., and Nyborg, J. (2000) The protein biosynthesis elongation cycle. In: Sonenberg, N., Hershey, J. W. B., and Mathews, M. B. (eds). Translational Control of Gene Expression, Cold Spring Harbor Laboratory, Cold Spring Harbor
10. Zavialov, A. V., Hauryliuk, V. V., and Ehrenberg, M. (2005) J. Biol. 4 :9
11. Olarewaju, O., Ortiz, P. A., Chowdhury, W., Chatterjee, I., and Kinzy, T. G. (2004) RNA Biology 1, 12-17
12. Andersen, G. R., Pedersen, L., Valente, L., Chatterjee, I., Kinzy, T. G., Kjeldgaard, M., and Nyborg, J. (2000) Mol. Cell 6, 1261-1266
13. Pittman, Y., Valente, L., Jeppesen, G. R., Andersen, G. R., and Patel, S. (2006) J. Biol. Chem. 281(28), 19457-19468
14. Carr-Schmid, A., Valente, L., Loik, V. I., Williams, T., Starita, L. M., and Kinzy, T. G. (1999) Mol. Cell. Biol. 19, 5257-5266
15. Schirmaier, F., and Phillipson, P. (1984) EMBO 3, 3311-3315
16. Anand, M., Valente, L., Carr-Schmid, A., Munshi, R., Olarewaju, O., Ortiz, P., and Kinzy, T. G. (2001) Symposium in Quantitative Biology 46, 439-448
17. Hiraga, K., Suzuki, K., Tsuchiya, E., and Miyakawa, T. (1993) FEBS Lett. 316, 165-169
18. Kinzy, T. G., and Woolford, J. L., Jr. (1995) Genetics 141, 481-489
19. Ozturk, S. B., Vishnu, M. R., Olarewaju, O., Starita, L. M., Masison, D. C., and Kinzy, T. G. (2006) Genetics 174(2)(2), 651-663
FIGURE LEGENDS

**Fig. 1.** GEF-independent mutants of eEF1A reduce GDP binding. Equilibrium dissociation constants ($K_d$) for the mutant forms of eEF1A and mantGDP were measured. Aliquots of mantGDP were added to binding buffer without (A) or with 1µM wild type (B) or the mutant forms (C-E) of eEF1A. The fluorescence was measured by FRET via excitation at 280 nm and emission of 440 nm for mant moiety. Fluorescence intensity (cpm) versus the concentration of mantGDP (µM) was plotted and data (●) was fit to a hyperbolic curve to obtain the $K_d$ value. The $K_d$ values are measured as 3.54 (T22S, C), 2.66 (R164K, D), 1.80 (A112T, E) and 1.18 µM (A117V, F). Residuals for the fits are shown in the lower panels to detect the experimental error for the fitted data sets.

**Fig. 2.** GEF-independent mutants of eEF1A do not affect GMPPNP binding. The $K_d$ values for the wild type and mutant forms of eEF1A and mantGMPPNP were measured. Aliquots of mantGMPPNP were added to binding buffer without (A) or with 1µM of wild type (B) or mutant forms (C-E) of eEF1A. The fluorescence was measured and plotted as in Figure 1. The $K_d$ values are measured as 0.47 (T22S, C), 2.66 (R164K, D), 1.80 (A112T, E) and 1.18 µM (A117V, F). Residuals for the fits are shown in the lower panels to detect the experimental error for the fitted data sets.
Fig. 3. Mutant forms of eEF1A demonstrate faster GDP dissociation. GDP dissociation rate constants (k) for the wild type and the mutant forms of eEF1A were measured. Mutant forms of eEF1A (A-D) prebound to mantGDP were rapidly mixed with excess GDP. The rate of mant nucleotide release was monitored as a decrease in fluorescent intensity over time and fitted by a single-exponential decay equation to obtain $k_{off}$ values. The $k_{off}$ values are measured as 3.89 (A117V, A), 2.54 (R164K, B), 1.96 (A112T, C) and 1.88 (T22S, D) $s^{-1}$. Residuals for the fits are shown in the lower panels to detect the experimental error for the fitted data sets.

Fig. 4. Mutant forms of eEF1A show enhanced thermostability compared to the wild type protein. The wavelength spectrums of the 0.2-0.4 mg/mL of purified proteins are carried out at the indicated temperatures. (A) Circular Dichroism spectra of wild type eEF1A (●, ○) and A112T (▲, △) forms of eEF1A. Closed symbols represent the wavelength spectrum at the native state (25°C) while open symbols represent the spectrum at 70°C. (B) Circular Dichroism spectra of the mutant forms of eEF1A, R146K (○), T22S (▲), A112T (△) and A117V (■), in comparison to wild type (●) at 70°C.

Fig. 5. GEF-independent mutants increase eEF1A stability. Unfolding intermediates of the wild type (A), A112T (B), R164K (C), A117V (D) and T22S (E) forms of eEF1A measured by Circular Dichroism spectra and collected as a function of temperature (left panel). The spectra were deconvoluted into three basis curves using the CCA. Each curve (■), (△) and (○) represents a different state of unfolding. Right panel shows the fraction of each basis curve contributing to each spectrum at each temperature.

Fig. 6. Guanine nucleotides increase eEF1A stability. Circular Dichroism spectra of apo eEF1A (A), eEF1A with GDP (B) and eEF1A with GMPPNP (C) were measured. The spectra were deconvoluted into three basis curves using the CCA. Each curve (■), (△) and (○) represents a different state of unfolding.

Fig. 7. GEF-independent mutants of eEF1A either affect the P-loop or the NKXD element. (A) T22 (magenta) and A112 (cyan) residues and their positions respective to the nucleotide (green) and the P-loop (magenta) are shown. (B) R164 (wheat) and A117 (purple) residues and their positions respective to the nucleotide (green) and the NKXD motif (yellow) are shown. The structure was produced with the PyMOL program (32) using the co-crystal structure of the eEF1A: eEF1Bα complex with GDP (PDB, 1IJE) (39). (C) Sequence alignment of yeast eEF1A with the G-proteins independent of GEF in yeast (eEF2, eIF5B, eRF3, Guf1p and Hbs1p) and human (eEF2, eIF5B, eRF3, Guf1p, Hbs1 like eRFs and eEFSec). The alignment was generated by Jalview (41).
| Strain  | Genotype                                                                 | Reference |
|---------|---------------------------------------------------------------------------|-----------|
| MC213   | MATα ura3-52 leu2-3,112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3 | (19)      |
| TKY368  | MATα ura3-52 leu2-Δ1 trp1-Δ101 lys2-801 met2-1 his4-713 tef5::TRP1 pTEF5 LEU2 | (13)      |
| TKY961  | MATα ura3-52 leu2-3,112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3 (R164K) | (19)      |
| TKY963  | MATα ura3-52 leu2-3,112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3 (T22S) | (19)      |
| TKY964  | MATα ura3-52 leu2-3,112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3 (A112T) | (19)      |
| TKY965  | MATα ura3-52 leu2-3,112 trp1-Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3 (A117V) | (19)      |
Table 2. $K_d$ values for mGDP and GTP, $k_{off}$ for mGDP.

|          | $K_d$ for mGDP (µM) | $K_d$ for mGMPPNP (µM) | $k_{off}$ (s$^{-1}$) for mGDP |
|----------|---------------------|------------------------|-------------------------------|
| Wild Type| 0.095+/-0.01        | 0.52+/-0.02            | 0.17                          |
| T22S     | 3.54+/-0.54         | 0.47+/-0.19            | 1.88                          |
| R164K    | 2.66+/-0.0          | 0.41+/-0.40            | 2.54                          |
| A112T    | 1.80+/-0.09         | 1.01+/-0.31            | 1.96                          |
| A117V    | 1.18+/-0.29         | 1.09+/-0.09            | 3.89                          |
Table 3. Analyses of the structure of wild type and the mutant forms of eEF1A from the CD data in native (N) and denatured (D) states of the proteins.*

| eEF1A forms | Helix total | β total | Turns | Other |
|-------------|-------------|---------|-------|-------|
|             | N  D        | N  D    | N    | D    | N    | D    |
| Wild Type   | 25 14       | 23 35   | 18    | 19   | 39   | 45   |
| A112T       | 24 21       | 23 26   | 18    | 19   | 40   | 43   |
| A117V       | 24 20       | 23 25   | 18    | 20   | 40   | 43   |
| T22S        | 24 18       | 23 28   | 18    | 20   | 40   | 46   |
| R164K       | 24 17       | 23 29   | 18    | 20   | 40   | 46   |

*The numbers represent percentage of each secondary structure.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Guanine nucleotide exchange factor independence of the G-protein eEF1A through novel mutant forms and biochemical properties
Sedide B. Ozturk and Terri Goss Kinzy

J. Biol. Chem. published online June 18, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M801095200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts