Translation Elongation Factor 2 Anticodon Mimicry Domain Mutants Affect Fidelity and Diphtheria Toxin Resistance*

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Eukaryotic elongation factor 2 (eEF2) mediates translocation in protein synthesis. The molecular mimicry model proposes that the tip of domain IV mimics the anticodon loop of tRNA. His-699 in this region is post-translationally modified to diphthamide, the target for Corynebacterium diphtheriae and Pseudomonas aeruginosa toxins. ADP-ribosylation by these toxins inhibits eEF2 function causing cell death. Mutagenesis of the tip of domain IV was used to assess both functions. A H694A mutant strain was non-functional, whereas D696A, I698A, and H699N strains conferred conditional growth defects, sensitivity to translation inhibitors, and decreased total translation in vivo.

These mutant strains and those lacking diphthamide modifications enzymes showed increased −1 frameshifting. The effects are not due to reduced protein levels, ribosome binding, or GTP hydrolysis. Functional eEF2 forms substituted in domain IV confer dominant diphtheria toxin resistance, which correlates with an in vivo effect on translation-linked phenotypes. These results provide a new mechanism in which the translational machinery maintains the accurate production of proteins, establishes a role for the diphthamide modification, and provides evidence of the ability to suppress the lethal effect of a toxin targeted to eEF2.

The eukaryotic translation elongation factor 2 (eEF2) and its bacterial homolog elongation factor G (EF-G) are members of the G-protein superfamily. These two proteins catalyze the translocation step of translation elongation after peptide bond formation occurs. The tRNAs located in the A- and P-sites are translocated to the P- and E-sites followed by the advancement of three bases of the mRNA to allow another round of translation elongation (reviewed in Ref. 1). In the yeast Saccharomyces cerevisiae, eEF2 is encoded by two genes, EFT1 and EFT2. The encoded proteins are identical, and one must be present for viability (2).

Even though work on EF-G has proven to have been invaluable in our understanding of the function of eEF2 on protein synthesis, marked differences are evident between the two homologous proteins. The most pronounced are the post-translation modifications that occur on eEF2. These modifications are the phosphorylation of Thr-57 and the diphthamide modification of His-699 in yeast and His-715 in mammals. S. cerevisiae eEF2 is phosphorylated by the Rck2p kinase (3), a Ser/Thr protein kinase homologous to the mammalian calmodulin kinases, which requires phosphorylation for activation (4, 5). In mammalian cells, eEF2 is phosphorylated on Thr-57 by the eEF2 kinase, a Ca2+/calmodulin-dependent protein kinase (6). The unique diphthamide modification is the result of a multistep conversion requiring several enzymatic activities performed by the DPH gene products in yeast (7). This modification is located at the tip of domain IV of the protein (8), a region proposed to mimic the tRNA anticodon loop (reviewed in Ref. 9). Although phosphorylation reduces the affinity for GTP, but not GDP, and decreases ribosome binding (10), a role for the diphthamide modification is unknown other than to serve as the site for ADP-ribosylation by the Corynebacterium diphtheriae diphtheria toxin (DT) and Pseudomonas aeruginosa exotoxin A (11). The action of either toxin inactivates the protein, whereas mutagenesis of His-699 demonstrated alterations of this residue are lethal or confer conditional growth defects and inhibit ADP-ribosylation (12, 13).

Crystal structures of the ternary complex EF-Tu-GTP-ami-noacyl-tRNA (14) and EF-G (15, 16) had suggested molecular mimicry between domains III, IV, and V of EF-G and tRNA (reviewed in Ref. 9). This suggests a close proximity of the tip of domain IV, the anticodon mimicry site, to the decoding site. The cryo-electron microscopic reconstruction of the yeast ribosome with eEF2 stabilized by the fungicide sordarin, an inhibitor of translocation in fungi that prevents eEF2 release (17), shows the tip of domain IV of eEF2 is in close proximity with the tRNA in the P-site (18). Residues 694–698 of eEF2 were modeled to be close enough to interact with the tRNA located in the P-site. Based on these results it was hypothesized that the tip of domain IV is involved in preventing errors in fidelity, in particular a −1 frameshift (18). We previously identified a possible relationship between this region of eEF2 and fidelity, because the presence of the H699K eEF2 mutant in a strain of yeast with wild-type eEF2 shows dominant sensitivity to the translation inhibitor paromomycin (19). Altered paromomycin sensitivity has been linked to errors in fidelity (20, 21). All these data support a role for the tip of domain IV, and likely the diphthamide modification, in eEF2 function.

Utilizing the available structural data proposing an important role for the anticodon mimicry domain of eEF2 in fidelity,
structure-based mutagenesis of residues 694, 696, 698, and 699 was performed. Mutants strains show increased sensitivity to translation inhibitors, a reduction of total translation, increased read-through of a programmed −1 frameshift signal, and resistance to diphtheria toxin. Strains with deletions in the DPH2 gene, which produces eEF2 with an unmodified His-699, or DPH5, which produces eEF2 with an intermediate modification, also show increased −1 frameshifting. Co-expression of the DT-resistant forms of eEF2 in the presence of the wild-type protein allows dominant prevention of the lethality conferred by the toxin expression. These results demonstrate the involvement of eEF2 preventing −1 frameshifting errors and provides an invaluable tool for the in vivo study of the consequences of ADP-ribosylation.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth**—*S. cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli* DH5α cells were used for plasmid preparation. Standard yeast genetic methods were employed (22). Yeast cells were grown in either YEPD (1% Bacto yeast extract, 2% peptone, and 2% dextrose) or defined synthetic complete media (C) or complete media lacking amino acids or nucleotides (C−) supplemented with 2% dextrose or 2% galactose as the carbon source (23). Yeast strains were transformed by the lithium acetate method (24). Growth was assayed by streaking on solid media or by growing 5-mL cultures of each strain overnight and diluting them to an A₆₀₀ of 1.0. Temperature sensitivity was assayed by spotting 10-fold serial dilutions (10 μL each) of the strains on YEPD followed by incubation at 13, 24, 30, and 37 °C for 2–7 days. The diphtheria toxin catalytic fragment was expressed from plasmid pLMY101 (25).

**DNA Manupulations and Mutagenesis**—Recombinant DNA techniques were performed as described (26). Restriction endonucleases and DNA-modifying enzymes were obtained from Roche or Invitrogen. Mutations in *eEF2* were created utilizing the PCR-based QuikChange site-directed mutagenesis kit (Stratagene). pTKB789 (eEF2<sup>His</sup><sup>H699A</sup>) was created using primers 5′-H699A (5′-CATTCTTGTATCTCCAGACG-3′) and 3′-H699A (5′-CCTGTTGAGTACCTGCGGACGTAAATGAC-3′), and pTKB703 (eEF2<sup>His</sup><sup>H699N</sup>) with primers 5′-H699N (5′-CATGCGATGTATCAACACAGAGGTGTGTGCAAACTC-3′) and 3′-H699N (5′-GATTGGACCCAAGACCTCGTTGTAGACACCGCATG-3′). All mutations were confirmed by restriction digestion and DNA sequence analysis.

**Protein Synthesis and Translation Inhibitor Sensitivity Assays**—For in vivo [35S]methionine incorporation and halo assays for sensitivity to cycloheximide, paromomycin, and hygromycin B were performed as previously described (38). Microtiter assays in liquid culture were performed for at least three independent colonies of each strain grown at 30 °C in liquid C-Leu media to mid-log phase, diluted to an A₆₀₀ of 0.1, and grown at 30 °C in triplicate in 96-well microtiter plates with varying concentrations of the translation inhibitor. Growth was monitored on a Bio-Tek Elx 800 microtiter reader and reported as the mean of the triplicate A₆₀₀ at 24 h.

**Western Blot Analysis and Ni-NTA Binding Assay**—Cells extracts were prepared as previously described (35). Total protein was determined by Bradford protein analysis (Bio-Rad), and 1 μg was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed with polyclonal antibodies to yeast eEF2 (1:20,000 dilution) or Pgk1p (1:10,000 dilution) and detected by a secondary antibody conjugated to peroxidase (1:7,500 dilution, Amersham Biosciences ECL plus). For the binding assay strains grown to mid-log phase were lysed in buffer A (50 mM potassium phosphate, pH 7.7, 300 mM KCl, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride) with 10 mM imidazole and lysed with glass beads. 30 μg of extract was incubated with Ni-NTA beads at 4 °C for 90 min with constant rocking, and the pellet was collected by centrifugation. The beads were washed three times with buffer A with 20 mM imidazole, boiled in loading buffer, subjected to SDS-PAGE, and immunoblotted with the eEF2 yeast polyclonal antibody.

**eEF2<sup>His</sup> Protein and 80 S Ribosome Purification**—To purify eEF2<sup>His</sup> from yeast, 4 liters of each strain was grown in YEPD to an A₆₀₀ of 1.5. Cells were harvested by centrifugation, suspended in buffer B (50 mM potassium phosphate, pH 8.0, 1 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 1% Tween 20) with 10 mM imidazole and lysed in a cell fluidizer (Microfluidics). After lysis the pH of the lysate was adjusted to 7.7 with 1 M Tris base and clarified by centrifugation at 12,500 rpm for 20 min. The supernatant was centrifuged at 50,000 rpm for 1 h, filtered through a 0.22-μm filter, and applied to an Amersham Biosciences Pharmacia His Trap Chelating Sepharose HP 3×1
column using an AKTA fast-protein liquid chromatography system. After washing with 5 volumes of buffer B with 20 mM imidazole, the protein was eluted with buffer B plus 500 mM imidazole. Pooled fractions were dialyzed overnight into buffer C (100 mM KCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8, 10% glycerol, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride). Yeast 80 S ribosomes were purified as described previously (28) from BJ3505 grown in YE PD to an A_{260} of 2.

Ribosome Binding Analysis—Ribosome binding was measured as described in Ref. 29 with minor modifications. 50-μl reactions containing various concentrations of eEF2 (2.5, 10, and 20 pmol) and 33 pmol of 80 S ribosomes in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM ammonium acetate, 10 mM magnesium acetate, 2 mM DTT, and 100 μM GDPNP) were incubated for 5 min at room temperature, layered on top of a 200-μl sucrose cushion (10% sucrose in binding buffer) and centrifuged at 204,000 × g for 20 min at 4 °C in a S80-AT2 (Sorvall) rotor. Pellets (bound fraction) were resuspended in Laemmli loading buffer, subjected to SDS-PAGE, and stained with gel code blue (Pierce). The amount of bound eEF2 was quantified with ImageJ software (National Institutes of Health).

GTPase Assay—eEF2 GTP hydrolysis activity was monitored with the method of Ref. 30 with minor modifications. 20-μl reactions containing 10 pmol of eEF2, 10 pmol of 80 S ribosomes, 100 μM GTP (containing 76 pmol of [γ-32P]GTP (6000 Ci/mmol) and GTPase buffer (25 mM Tris-HCl, pH 7.5, 125 mM KCl, 8.5 mM MgCl₂, 1 mM DTT, 6.25% glycerol) were incubated for 15 min at 37 °C and then placed on ice. The following additions were then made in order: 0.5 ml of silicic acid in 20 mM H₂SO₄; 1.2 ml of K₂HPO₄, pH 7.0; 0.5 ml of 5% ammonium molybdate in 4M H₂SO₄; and 5% trichloroacetic:acetone (1:1). Free γ-32P was extracted with isobutanol:benzene (1:1) by vortexing for 30 s followed by centrifugation for 3 min at 1500 rpm. One milliliter of organic phase was mixed with EcoLume scintillation fluid (ICN), and the amount of released [γ-32P]GTP was measured in a liquid scintillation counter.

Translational Fidelity and Killer Virus Assays—Strains were transformed with theURA3 wild-type lacZ control plasmid pJD204.0 or URA3 plasmids containing lacZ with programmed in-frame −1 frameshift (pJD204.−1) or +1 frameshift (pJD204.+1) signals (31) or URA3 wild-type lacZ control plasmid (pUKC815tail) or URA3 plasmids with in-frame UAA (pUKC817tail), UAG (pUKC818tail), and UGA (pUKC819tail) nonsense codons in lacZ (32) and assayed as previously described (33). Strains were cytoduced using the kar1-1 mutant JD759 containing L-A and M₁ and assayed for killing of 5′ to 47 M₁ toxin-sensitive cells as described (33).

Mass Spectrometry—Protein samples were denatured in guanidine-HCl, reduced using DTT, alkylated with iodoacetamide, buffer exchanged into 50 mM ammonium bicarbonate, and digested with trypsin using conventional procedures. Chromatography was conducted using an ultimate nano-LC system (Dionex/LC Packings) and a fritless nanoscale column (75 μm × 15 cm) packed-in-house with Poros R10, 2% (Applied Biosystems). The column was equilibrated in 0.1% formic acid (Solvent A), and samples were eluted using a linear gradient from 2% to 45% solvent B (0.1% formic acid in acetonitrile) over 30 min at a flow rate of 200 nl/min and analyzed by electrospray ionization-MS/MS using an LTQ ion trap mass spectrometer (ThermoFinnigan) equipped with a nanospray source (Proxeon Biosystems). Each MS scan was followed by subsequent zoom scans and MS/MS scans of the four most abundant multiply charged ions, with a dynamic exclusion time of 1 min. DTA files for MS/MS spectra were generated by Bioworks software (ThermoFinnigan) and searched against a yeast data base (NCBI nr) using Sequest (34) for preliminary assignments. Spectra were further analyzed by manual inspection.

RESULTS

Mutant Strains Expressing Forms of eEF2 with Alterations in the Tip of Domain IV confer Growth Phenotypes—The anticodon mimicry domain IV of eEF2 is the site of the unique post-translational modification of diphtheramide. The tip of domain IV of eEF2 is in close proximity to the decoding site of the ribosome in the cryo-electron microscopic reconstruction of the yeast eEF2-ribosome complex in the presence of sordarin (18). To address the functional significance of this region in vivo and in vitro we have targeted a series of residues for mutagenesis. The residues have been selected based on the x-ray crystal structure of eEF2 (8) and the cryo-electron microscopic recon-
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Figure 2. Altered domain IV forms of eEF2 compromise function in vivo. A, YEFD12b transformed with pRS315 (empty), pTKB612 (eEF2-HIS), or TKB709 (eEF2-HIS_H694A) was streaked on C-Leu or 5-fluoroorotic acid plates and incubated for 3–7 days at 30 °C. B, protein extracts were prepared from transformants in A grown to mid-log phase at 30 °C, an Ni-NTA binding assay was performed, and bound proteins were resolved by SDS-PAGE and detected with an antibody against eEF2. Lanes are pellet (P) and supernatant (S). C, strains expressing wild-type eEF2_HIS (TKY675), eEF2_HIS_H694A, eEF2_HIS_H699N (TKY825), eEF2_HIS_H699A, eEF2_HIS_H699B (TKY826), or eEF2_HIS_H699B (TKY742) were grown to mid-log phase at 30 °C in YEPD media, spotted as 10-fold serial dilutions on YEPD plates, and incubated at 30 and 37 °C for 3 days. D, strains from C were grown to mid-log phase at 30 °C, and total proteins were extracted (left panel) or transferred to 37 °C and grown for 6 h followed by protein extraction (right panel). The protein extracts were resolved by SDS-PAGE and detected with antibodies against eEF2 and Pgk1p (loading control).

The lack of function of H694A was not based on the lack of expression, as shown by Ni-NTA pull-down assay (Fig. 2B) performed due to the inability of an anti-His antibody to recognize the mutant proteins, as observed by Western blot analysis with the eEF2 antibody of extracts of cells grown at 30 or 37 °C (Fig. 2D).

Domain IV Mutant Strains Show Translational Defects—To determine if the observed growth phenotypes are linked to defects in total translation, a [35S]methionine incorporation assay was performed (Fig. 3). The analysis showed that the mutant strains that conferred a severe Ts− phenotype also reduced total translation at the permissive temperature by 35% (H699N) and 50% (D696A). Protein synthesis defects were further analyzed by utilizing the translation elongation inhibitors cycloheximide, paromomycin, and hygromycin B. Co-expression of wild-type and non-functional H694A (data not shown) or a strain with I698A alone (Table 2) showed no changes in sensitivity or resistance to the inhibitors as monitored by a halo assay. Mutant strains expressing the H699N or D696A forms of eEF2 showed paromomycin and hygromycin B resistance of 82 and 25%, respectively (Table 2). Altered paromomycin sensitivity was quantitated by a liquid microtiter growth assay in the presence of increasing concentrations of the inhibitor. Consistent with the observation in the halo assay, eEF2_H699A showed no change in the LC50 for paromomycin, whereas the LC50 observed for eEF2_D696A was reduced 46 and 65%, respectively (Table 2). Altered paromomycin sensitivity correlates with altered translational fidelity in S. cerevisiae (20, 21), indicating these strains may have a defect in their accuracy of protein synthesis.

Domain IV Mutant Strains Show Decreased Translational Fidelity via a −1 Frameshift—Based on the sensitivity observed against paromomycin, translational accuracy of the mutants eEF2_HIS (19). This result is not a surprise due to the identity of this residue in all prokaryotes and eukaryotes (Fig. 1B). Co-expression of H694A with the wild-type protein resulted in a dominant negative growth phenotype (Fig. 2A), similar to the results seen for the co-expression of other non-functional forms of eEF2 that reduce the pool of functional eEF2 (19). The D696A, I698A, and H699N forms of eEF2 allowed for the loss of a plasmid expressing wild-type eEF2 (Fig. 2C). A strain expressing D696A showed a slow growth phenotype at the permissive temperature (Fig. 2C, left panel). When growth was monitored at the non-permissive temperature of 37 °C, the I698A strain showed a slight decrease in growth, whereas D696A and H699N strains resulted in inviability (Fig. 2C, right panel). The growth defects were not due to a decrease in expression of the mutant proteins, as observed by Western blot analysis with the eEF2 antibody of extracts of cells grown at 30 or 37 °C (Fig. 2D).

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they could produce the toxin and kill sensitive cells. The ability were cytoduced into the wild-type and mutant strains such that eEF2HIS
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programmed on nonsense suppression at all three stop codons as well as strains was assessed. Mutant strains were analyzed for effects on nonsense suppression at all three stop codons as well as constructs containing lacZ in-frame stop codons or programmed +1 and −1 frameshifts followed by analysis of the production of β-Gal. Nonsense and +1 frameshifting were unaffected (data not shown), whereas the frequency of a −1 frameshift was increased in all the mutant strains (Fig. 4A). The I698A mutation was found to most dramatically affect −1 frameshifting. This is to our knowledge the first mutation to confer a fidelity error without showing altered resistance or sensitivity to paromomycin.

To determine if the increase in programmed −1 frameshifting observed in the lacZ constructs correlates with altered viral maintenance, we have taken advantage of the yeast L-A double-stranded DNA virus. The L-A virus has a satellite virus M1, which produces a toxin, but to propagate M1 efficiently the −1 frameshift must not be altered (35). The L-A and M1 viruses were cytoduced into the wild-type and mutant strains such that they could produce the toxin and kill sensitive cells. The ability of the wild-type and mutant strains to propagate M1 was mon-

Diphthamide Modification Enhances the Fidelity of Translation at a −1 Frameshift—The function of the diphthamide modification of His-699 in eEF2 is not known. Because alterations in the tip of domain IV, including His-699, showed an increase in −1 frameshifting, we hypothesized that the loss of the diphthamide modification could also have this phenotype. The H699N form of eEF2 cannot be ADP-ribosylated (13) and showed increased −1 frameshifting. To remove the diphthamide modification without changing the side chain we utilized strains that carry deletions in two DPH genes. Deletions of these genes inhibit the diphthamide modification on eEF2 (7, 25, 36). Strains carrying deletions of the DPH2 or DPH5 genes do not confer growth defects or sensitivity to the translation inhibitors cycloheximide and hygromycin B (data not shown). Paromomycin sensitivity cannot be assayed due to the KanMX cassette used to disrupt the genes, which confers resistance to the drug. Similar to the H699N mutant strain, the DPH2 and DPH5 deleted strains did not show an effect on nonsense suppression or +1 frameshifting (data not shown), whereas increased −1 frameshifting was observed for both (Fig. 4B). These strains did not show effects on viral maintenance as measured by the killer assay, likely due to modest −1 frameshift effect (data not shown). These results show that eEF2 with an unmodified or partially modified His-699 do not show drastic growth phenotypes but increase −1 frameshifting.

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trated by measuring the halo killing around spots of the eEF2 strains on a lawn of sensitive cells. The assay showed a slight decrease in the killing zone in the eEF2 mutant cells, indicating that viral maintenance has been compromised (data not shown).

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FIGURE 3. eEF2HISD506A and eEF2HISH699N strains show a decrease in total protein synthesis. Strains expressing wild-type eEF2HIS (TKY675, diamonds), eEF2HISD506A (TKY825, triangles), eEF2HISD696A (TKY826, X), or eEF2HISH699N (TKY742, squares) were grown in C-Met medium to mid-log phase at 30 °C. [35S]Methionine was added, and total protein synthesis was measured at each time point by trichloroacetic acid precipitation. Incorporation (in counts per minute) is expressed per A600 unit.

Strains expressing altered forms of eEF2

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A

B

FIGURE 4. Alterations in domain IV caused increased −1 frameshifting. Strains containing wild-type eEF2HIS (TKY675), eEF2HISD506A (TKY825), eEF2HISD696A (TKY826), or eEF2HISH699N (TKY742) (4) or wild-type (BY4742), Δdph2, or Δdph5 (8) strains were transformed with URA3 wild-type lacZ control plasmid pJD204.0 or an in-frame −1 frameshift in lacZ pJD204.−1 and assayed for β-galactosidase production by the O-nitrophenol-β-galactopyranosidase assay. Frameshifting (FS) is expressed relative to the level in an isogenic wild-type strain.

sensitivity to paromomycin. The H699N form of eEF2 cannot be ADP-ribosylated (13) and showed increased −1 frameshifting. To remove the diphthamide modification without changing the side chain we utilized strains that carry deletions in two DPH genes. Deletions of these genes inhibit the diphthamide modification on eEF2 (7, 25, 36). Strains carrying deletions of the DPH2 or DPH5 genes do not confer growth defects or sensitivity to the translation inhibitors cycloheximide and hygromycin B (data not shown). Paromomycin sensitivity cannot be assayed due to the KanMX cassette used to disrupt the genes, which confers resistance to the drug. Similar to the H699N mutant strain, the DPH2 and DPH5 deleted strains did not show an effect on nonsense suppression or +1 frameshifting (data not shown), whereas increased −1 frameshifting was observed for both (Fig. 4B). These strains did not show effects on viral maintenance as measured by the killer assay, likely due to modest −1 frameshift effect (data not shown). These results show that eEF2 with an unmodified or partially modified His-699 do not show drastic growth phenotypes but increase −1 frameshifting.
Forms of eEF2 Altered in Domain IV Do Not Affect eEF2 GTP Hydrolysis or Ribosome Binding—To investigate if the phenotypes seen for the eEF2 mutant strains are the result of specific deficiencies in protein function, the altered eEF2 forms were affinity-purified from yeast and mechanistic effects analyzed. Ribosome binding effects were analyzed by a sucrose cushion binding method (29). Following incubation the ribosome-eEF2 complex was placed on a 10% sucrose cushion, ribosome-bound eEF2 pelleted and binding analyzed by SDS-PAGE (Fig. 5A). The binding assay showed that the mutants did not affect ribosome binding. As GTase activity is essential for the function of eEF2, the effects of the mutants on ribosome-dependent GTP hydrolysis was determined by an in vitro GTP hydrolysis assay (30) (Fig. 5B). The results show that the mutations do not significantly affect ribosome-stimulated eEF2 GTase activity. Thus, although the tip of domain IV inserts into the ribosomal A-site, mutations in this region do not affect ribosome binding or the transfer of the GTase signal.

Domain IV Mutant Strains Are Resistant to Diphtheria Toxin—The crystal structure of exotoxin A ADP-ribosylated eEF2 demonstrated that the residues selected in this study make contacts with the ribose attached to diphthamide (37). Residues His-694 and Asp-696 form a triangle of hydrogen bonds with the 2'-OH of the ribose attached to diphthamide, whereas Asp-696 interacts with the 3'-OH of the ribose. Although Ile-698 does not make any contacts with the ribose or diphthamide residue, it is between the interactions with the other residues and could affect them indirectly. Thus, it is possible that these mutants could interfere with ADP-ribosylation by diphtheria toxin (DT) or P. aeruginosa exotoxin A toxin. To observe the effect of the mutations on ADP-ribosylation in vivo, a galactose-inducible construct expressing the catalytic domain of DT was utilized (25). The DT construct was transformed into wild-type and mutant strains, and ADP-ribosylation was assayed by death on galactose media when the toxin is expressed. Strains carrying the D696A, I698A, and H699N eEF2 mutants survived in the presence of DT expression compared with a wild-type strain (Fig. 6A). DT expression and function were confirmed by transformation of the DT construct into strains carrying deletions in the DPH2 and DPH5 genes, which also survive growth on galactose media (Fig. 6B and data not shown).

Altered Domain IV Forms of eEF2 Confer Dominant Resistance to DT—Intracellular diphtheria toxin expression results in irreversible ADP-ribosylation of eEF2 and lethality. ADP-ribosylation of eEF2 does not affect ribosome or nucleotide binding, inferring an inhibition of function after the protein has bound the ribosome (37). In an effort to recover viability of strains expressing DT as a model for this pathology, plasmids expressing DT-resistant forms of eEF2 were transformed into a strain also bearing wild-type eEF2. Strains co-expressing wild-type and an I698A or H699N form of eEF2 were able to survive in the presence of DT expression on media containing galactose (Fig. 6B). A strain expressing D696A also shows modest growth, however, this mutant shows the most significant growth defect when present as the only form of eEF2. To determine if a cell expressing a resistant form of eEF2 and ADP-ribosylated eEF2 has effects on translation in vivo, we monitored the strains for altered sensitivity to translation inhibitors by the halo assay. The analyses compared a strain carrying a DT expression or empty plasmid, a plasmid expressing an altered form of eEF2, and chromosomal wild-type eEF2 between growth on glucose versus galactose media (Table 3). When grown on glucose or on galactose with an
empty vector the I698A of H699N mutant-expressing strains did not show sensitivity to any inhibitor (Table 3). Similar sensitivity was seen when H699N was the only form of eEF2 (Table 2). When sensitivity was determined in media containing galactose with DT expression, the defect caused by ADP-ribosylation on translation is evident (Table 3). DT expression caused increased sensitivity to all of the translation inhibitors. Thus, increased sensitivity appears to be the specific result of ADP-ribosylation and show indirectly that ADP-ribosylation has an effect in translation in vivo.

Mass Spectrometry of the Diphthamide Modification and Domain IV Mutants—To determine the mechanism of DT resistance, liquid chromatography-MS/MS was used to detect the presence or absence of diphthamide and its biosynthetic intermediates, eEF2HIS purified from wild-type, dph2Δ, or dph5A yeast strains, were analyzed by liquid chromatography-MS/MS. Data base searches were performed allowing potential modifications on histidine of +143 (diphthamide), +142 (diphthine), and +101 (the intermediate). For eEF2HIS purified from the dph2Δ strain, a peptide corresponding to unmodified tryptic fragment WTVLHADAIHR of eEF2HIS was reliably identified by MS/MS analysis (Fig. 7A). No diphthamide, diphthine, or the intermediate were found, consistent with the phenotype of this mutant. Analysis of eEF2HIS purified from a dph5Δ strain revealed the presence of a small amount of unmodified tryptic peptide 686–700 as well as substantial amounts of the peptide with a +101 modification (the intermediate). MS/MS analysis of the modified peptide yielded an ion series that was consistent with the peptide being modified at His-699 with the diphthamide biosynthetic intermediate that has been reported to accumulate in dph5Δ strain (7) (Fig. 7B). For eEF2HIS purified from wild-type yeast, only a trace amount of unmodified 686–700 peptide was found. In contrast, we observed substantial amounts of this peptide with a +143 modification (diphthamide) that eluted as two peaks at 33 and 37 min, respectively, under our chromatography condition. Fragmentation of the parent ions in both peaks yielded indistinguishable MS/MS spectra. For the doubly charged parent ion, the dominant product ion was the parent ion with a neutral loss of 58 Da (data not shown). Fragmentation of the triply charged parent ion yielded product ions with a neutral loss of 58 Da ([M + 3H]3+*, Fig. 7C), a fragment with a loss of a 59-Da positively charged group ([M + 2H]2+*, Fig. 7C), a series of doubly charged y and b ions, and doubly charged y13, y12, and y11 ions with a further neutral loss of 58 Da (Fig. 7C, asterisks). This fragmentation pattern allows confident assignment of the site of diphthamide modification to His-699, with the neutral loss likely due to the loss of an -N(CH3)3 group. The neutral loss mass signature might be helpful as a guide for identification of diphthamide-modified peptides by mass spectrometry. The appearance of two liquid chromatography-separated populations with identical m/z (judged by zoom scan) and un distinguishable MS/MS spectra sug-

**TABLE 3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose (no DT)</th>
<th>Galactose (DT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paromomycin</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>eEF2 Wt</td>
<td>800 ng/ml</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>eEF2 Wt + empty</td>
<td>10 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>eEF2 D696A</td>
<td>11 ± 1</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>eEF2D696A + DT</td>
<td>10 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>eEF2D696A + empty</td>
<td>9 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>eEF2D696A + DT</td>
<td>12 ± 1</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

*FIGURE 6. Altered domain IV forms of eEF2HIS confer recessive and dominant resistance to DT. A, strains containing wild-type eEF2HIS (TKY675), eEF2HIS D696A (TKY825), eEF2HIS I698A (TKY826), or eEF2HIS H699N (TKY742) and a URA3 plasmid with a galactose-inducible diphtheria toxin catalytic fragment (pLMY101) were streaked on C-Ura or C-Ura plus galactose plates and incubated for 3–7 days at 30 °C. B, BY4742 (EFT1 EFT2) containing a wild-type eEF2 plasmid was transformed with pLMY101 (DT) and an empty plasmid (pRS315), an eEF2HIS (pTKB612, Wt), eEF2HIS D696A (pTKB706), eEF2HIS I698A (pTKB709), or eEF2HIS H699N (pTKB703) plasmid. The negative control is BY4742 with pRS315 and pRS316 empty plasmids as well as pLMY101, and the positive control is a dph2Δ strain with pLMY101 and pRS315. All strains were streaked on C-Leu-Ura or C-Leu-Ura plus galactose plates and incubated for 3–7 days at 30 °C.*
Fidelity and Diphtheria Toxicity Effects of eEF2

A

Relative Abundance

VNILDVTLHADIHR

b⁺2 b⁺3

m/z

y⁺2⁺12

y⁺2⁺13

y⁺2⁺14

B

Relative Abundance

VNILDVTLHADIHR

INTERMEDIATE

b⁺2 b⁺3

y⁺2⁺11

y⁺2⁺14

C

Relative Abundance

[M+3H]⁺3⁺

VNILDVTLHADIHR

[Diphthamide]

b⁺2 b⁺3

y⁺2⁺12

y⁺2⁺11

y⁺2⁺13

[M+2H]⁺2⁺
The anticondon mimicry mutants show that this region is essential for eEF2 function, because an H694A form is inviable and strong conditional growth defects are observed for D696A, I698A, and H699N mutant strains. D696A and H699N mutant strains show decreased total translation and have sensitivity to translation inhibitors, suggesting their effects are at the elongation step of protein synthesis. The phenotypes observed by the mutants are not the result of a defect in ribosome binding or GTPase activity. Mutagenesis studies performed on the bacterial homolog EF-G have shown that deletion of domain IV or mutagenesis of His-583 (equivalent to yeast His-694) affects translational hydrolysis without affecting ribosome binding or GTP hydrolysis (40, 41). These results support the notion that altered forms of eEF2 cause a translational deficiency.

The unique diphthamide modification of His-699 in yeast (His-715 in mammals) is the result of a series of enzyme-catalyzed steps performed by the DPH gene products. Until now the role of this modification in eEF2 function was not well understood. Previous systematic mutagenesis of His-699 to all 19 amino acids inhibited formation of diphthamide, inhibited ADP-ribosylation, and caused varying growth defects (12, 13). The dominant effects are predominantly due to regulation of eEF2 levels and the reduced pool of active protein (19). Mutagenesis of Ser-584 to Gly (Asp-568 in S. cerevisiae), Ile-714 to Asn (Ile-698 in S. cerevisiae), and Gly-719 to Asp (Gly-703 in S. cerevisiae) in Chinese hamster ovary cells yielded unmodified eEF2 forms, which confer resistance against ADP-ribosylation and reduced protein synthesis (42). Here, the novel mutations that we have studied have yielded proteins that both confer resistance to diphtheria toxin expression and show defects in reading frame maintenance in vivo. To study the diphthamide modification without affecting the eEF2 protein sequence, we have taken advantage of strains deleted for genes required for diphthamide biosynthesis, DPH2 and DPH5. These deletion strains yield unmodified or partially modified forms of eEF2 but do not have growth defects or grossly affect translation as measured by their effect on translational inhibitors. During the study of more subtle effects, we have shown the role of the diphthamide modification in maintaining translational fidelity. Because these effects are significant but modest, these results cannot rule out additional roles for the diphthamide modification on eEF2 function such as protein stability.

The diphthamide modification is the only site for ADP-ribosylation by diphtheria toxin and exotoxin A in the cell. Even though much is known about the enzymes that ADP-ribosylate eEF2, the mechanism by which ADP-ribosylation inhibits eEF2 function is still unknown. ADP-ribosylation does not affect nucleotide or ribosome binding, suggesting inhibition after eEF2 has bound the ribosome (37). Our expression of the DT toxin-resistant forms of eEF2 in a wild-type strain carrying the EFT1 and EFT2 genes resulted in the recovery of viability of strains expressing DT. This result led us to the in vivo study of

**DISCUSSION**

The accuracy of fidelity in translating an mRNA into a protein is affected by cis- and trans-acting factors at all three steps of protein synthesis (38). During elongation, maintenance of the correct reading frame is critical. A shift in the decoding of the mRNA in the 5′ or 3′ direction can result in a truncated protein by encountering a premature stop codon and trigger outcomes such as nonsense-mediated mRNA decay. The involvement of eEF2 in the accurate production of proteins, a G-protein that catalyzes the translocation of tRNAs and the advancement of the mRNA during translation elongation, has been determined. Cryo-electron microscopic reconstruction of the yeast ribosome-eEF2 complex in the presence of sordarin shows that the tip of domain IV of eEF2, proposed as an anticodon mimic, is in close proximity to the tRNA located in the ribosomal P-site (18). Due to this placement it was hypothesized that eEF2 is involved in stabilizing codon-anticodon pairing during translocation, thus preventing a frameshift. A set of novel forms of eEF2 altered in the tip of domain IV, D696A, I698A, and H699N, demonstrated the hypothesized effect of eEF2 in the maintenance of the proper reading frame specific to −1 frameshifting. Previously, the Dinman group proposed an integrated model for programmed ribosomal frameshifting (39), describing −1 and +1 programmed ribosomal frameshifting effects due to the step of the translation elongation cycle and tRNA occupancy. In this model selection of the cognate tRNA and eEF2 translocation are involved in the prevention of a +1 frameshift, while accommodation of the tRNA and peptidyl transfer prevent a −1 frameshift. This effect of the translocation step was proposed based on the effects of a series of altered eEF2 forms selected for resistance to sordarin, all of which promoted +1 frameshifting and cluster in domain III. These mutants may affect +1 frameshifting by affecting the eEF2 function during or after translocation. The domain IV mutations studied here are located at the tip of domain IV, and the different effect observed in −1 frameshifting is consistent with effects on eEF2 function prior to translocation. Thus, our results extend the role of eEF2 to a more general role in the maintenance of the reading frame.

**FIGURE 7. Characterization of diphthamide biosynthesis by MS.** A, MS/MS of spectra of tryptic fragment Q686YNLVDTLHADAIH700 from eEF2HIS purified from a dph2 strain. B, MS/MS spectrum of the same peptide with modification of the intermediate at His-699 from eEF2HIS purified from a dph2 strain. C, MS/MS of tryptic fragment 686–700 from eEF2HIS purified from a wild-type strain. y and b ions are marked. Peaks are peaks of neutral loss of 58 Da.
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the effect of ADP-ribosylation using translational inhibitors. In strains expressing the toxin, sensitivity to translational inhibitors was increased, showing a correlation between ADP-ribosylation and translational defects in vivo. Thus, while DT is toxic, under conditions where viability is maintained by a resistant form of eEF2, the effects of DT on translation are apparent and allow for the further dissection of the mechanism of the effects of ADP-ribosylation. Clearly, the translation effect of ADP-ribosylation is not simply due to a reduced pool of active eEF2, because the expression of the non-functional eEF2 V488A form in the wild-type strain results in reduced wild-type eEF2 levels but does not result in sensitivity to translation inhibitors. Thus, these strains are a valuable resource in the study of ADP-ribosylated eEF2 in vivo. Our studies have shown that functional and non-functional eEF2His can be efficiently purified by affinity methods. Therefore, expression of our toxin-resistant forms of eEF2 in strains carrying the wild-type eEF2His provides an efficient system to affinity-purify ADP-ribosylated eEF2 for in vitro studies and to produce strains to study factors and compounds that inhibit ADP-ribosylation in vivo.

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