Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A

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Ribosomal translation factors are fundamental for protein synthesis and highly conserved in all kingdoms of life. The essential eukaryotic elongation factor 1A (eEF1A) delivers aminoacyl tRNAs to the A-site of the translating 80S ribosome. Several studies have revealed that eEF1A is posttranslationally modified. Using MS analysis, site-directed mutagenesis, and X-ray structural data analysis of Saccharomyces cerevisiae eEF1A, we identified a posttranslational modification in which the α amino group of mono-L-glutamine is covalently linked to the side chain of glutamate 45 in eEF1A. The MS analysis suggested that all eEF1A molecules are modified by this glutaminylation and that this posttranslational modification occurs at all stages of yeast growth. The mutational studies revealed that this glutaminylation is not essential for the normal functions of eEF1A in S. cerevisiae. However, eEF1A glutaminylation slightly reduced growth under antibiotic-induced translational stress conditions. Moreover, we identified the same posttranslational modification in eEF1A from Schizosaccharomyces pombe but not in various other eukaryotic organisms tested despite strict conservation of the Glu residue among these organisms. We therefore conclude that eEF1A glutaminylation is a yeast-specific posttranslational modification that appears to influence protein translation.

One of the most conserved biological processes is ribosomal protein synthesis, comprising initiation, elongation, termination, and recycling steps. Each step is dependent on specific translation factors. Eukaryotic elongation factor 1A (eEF1A), a large GTPase that is one of the most abundant cytosolic proteins, is important for the binding, stabilization, and delivery of aminoacylated tRNA to the translating ribosome. Correct codon–anticodon pairing of the aminoacyl–tRNA with the mRNA at the ribosomal A-site triggers ribosome-dependent hydrolysis of GTP and leads to dissociation of eEF1A from the ribosome. eEF1A is reactivated by nucleotide exchange factor eEF1B (1) and then able to reassociate with charged tRNA to start a new translation cycle. Besides its essential role in mRNA translation, eEF1A participates in many other cellular functions and is reportedly involved in actin bundling, nuclear export (2), signal transduction (3), apoptosis, proteasomal degradation, and tumorigenesis (4, 5).

Crystal structures of archaeal, mammalian, and yeast eEF1A (partially in complex with subunits of eEF1B) showed that the elongation factor consists of three domains (I–III) (6–8). Domain I is the GTP-binding domain and resembles large GTPases of the Ras family. Domains II and III are likely to act as a rigid functional unit and are involved in aminoacyl–tRNA binding. These domains were also reported to be implicated in the interaction with cytoskeletal proteins (9, 10).

eEF1A is extensively posttranslationally modified by lysyl acetylation, methylation (11), ubiquitination, nitrosylation, glutathionylation, phosphorylation (12), C-terminal methyl esterification (13), and the attachment of ethanolamine phosphoglycerol (EPG) (14). Most posttranslational modifications of eEF1A occur in domains II and III, whereas fewer modifications are found in the enzymatic GTPase domain. However, domain I of eEF1A is posttranslationally modified by Legionella pneumophila glucosyltransferases Lgt1–3, which attach glu-
cose onto Ser53 of yeast and mammalian eEF1A (15, 16).
Although toxin-induced modification of eEF1A results in inhibition of protein synthesis in yeast and mammalian cells, the roles and functional consequences of most endogenous posttranslational modification of eEF1A are still not clear (17).

eEF1A belongs to the most conserved proteins across all kingdoms of life (18). However, in contrast to the prokaryotic orthologue, eukaryotic and archaeal EF1A contain an additional subdomain, the helix A*-loop–helix A/H11032 region (amino acids 36–69). This double helix insert is part of the switch I region within the GTPase domain. Extensive structural alterations of this region during GDP/GTP exchange and during interaction with the ribosome suggest a pivotal role of this additional helix–loop–helix region in eukaryotic organisms (7).

Here we describe, for the first time, a novel type of posttranslational modification, the glutaminylation of a glutamate residue, that occurs within the helix A*-loop–helix A/H11032 region of yeast eEF1A. We show that the glutamine residue is attached via its carboxyl group to the side chain carboxyl group of Glu45 within eEF1A. Glutaminylation of eEF1A was detected in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Experiments with S. cerevisiae expressing Glu45 eEF1A mutants instead of the wild-type elongation factor indicate that glutaminylation slightly enhances growth defects under translational stress conditions.

Results

Posttranslational modification of the switch I region in eEF1A

We set out to specifically analyze the helix A*-loop–helix A’ region of EF1A for unconventional posttranslational modifications. eEF1A was purified from S. cerevisiae using affinity chromatography with His6-tagged guanine nucleotide exchange factor eEF1B (19). MS analysis revealed that the tryptic peptide 42-FEKEAAELGK-51 (M+2H)2+ = 625.3251 of yeast EF1A is modified at a side chain of Glu45. Immon., immonium ion; Seq., sequence.

Figure 1. eEF1A isolated from S. cerevisiae is posttranslationally modified in the GTPase domain at Glu45. A, extracted ion chromatogram showing that the tryptic peptide 42-FEKEAAELGK-51 of yeast EF1A is covalently modified by the attachment of a mass of 128.059 Da. B, MS-MS fragmentation analysis revealed that the tryptic peptide 42-FEKEAAELGK-51 of yeast EF1A is modified at a side chain of Glu45. Immon., immonium ion; Seq., sequence.

Confirmation of eEF1A glutaminylation at Glu45 by site-directed mutagenesis

To confirm the glutaminylation of Glu45 in eEF1A, we performed MS-MS analysis with the site-directed mutants eEF1A E45A and eEF1A E45D expressed in and isolated from S. cerevisiae (Fig. 2). Mutation of Glu45 to alanine prevented modification by glutamine. Similarly, the mutant eEF1A E45D with shortened side-chain carbonyl was not modified. Thus, glutaminylation seems to be highly specific for glutamate at position 45. Notably, using wild-type eEF1A, we were not able to identify unmodified eEF1A in yeast cells. Also, at different cell stages of yeast growth, eEF1A was always completely glutaminylated (supplemental Fig. 1).
**l-Glutamine is linked to Glu⁴⁵ via the α amino group**

To analyze glutaminylation in molecular detail and clarify how the glutaminylo moiety is attached, we used the 1.67-Å-resolution crystal structure of *S. cerevisiae* eEF1A in complex with the C-terminal catalytic domain of the exchange factor eEF1B (PDB code 1F60 (8)), which is the highest-resolution structure available for this protein in the Protein Data Bank. An unassigned electron density close to residue Glu⁴⁵ permitted addition of a glutaminyl moiety in a defined orientation (Fig. 3, A and B). The α amino group of the glutamine is covalently linked to the δ C atom of the carboxylic group of the Glu⁴⁵ side chain. This is a peptide bond–like connection with a characteristic planar geometry and a short C-N bond. The bond formation results in the loss of one oxygen atom of the carboxylic group from Glu⁴⁵ and equates to the release of a water molecule (Fig. 3C). The modification extends the glutamate side chain as a branched moiety, with the side chain and the carboxylic group of glutamine both exposed to the surface of the GTPase domain of eEF1A. Structure refinement comparing the fit of l- and D-glutamyl is a mechanism known for several proteins, e.g. in polyglutamylation of α and β tubulin (20), γ-glutamylitation in glutathione metabolism, or xenobiotics detoxification (21). In contrast, the attachment of a single glutamine via its α amino group has not been reported previously.

**Mouse, bovine, fish, insect, and archaeal eEF1A are not glutaminylated at Glu⁴⁵**

Glu⁴⁵ is highly conserved within the helix A⁺–loop–helix A⁻ region of eukaryotic eEF1A and also present in several archaeal EF1A molecules (Fig. 4). To determine whether glutaminylation of Glu⁴⁵ is also conserved, we isolated elongation factors from fission yeast (*S. pombe*), archaea (*Haloferax volcanii*), zebrafish (*Danio rerio*) ZF4 fibroblasts, bovine (*Bos taurus*) liver, mouse (*Mus musculus*) brain and liver, and wax moth larvae (*Galleria mellonella*). Domain II and domain III of eEF1A molecules (Fig. 4) were analyzed by LC/MS-MS. Identified peptidic fragments were well-assigned and clearly indicated that glutaminylation at Glu⁴⁵ was present in eEF1A from fission yeast (*S. pombe*) but not present in elongation factors isolated from any of the other organisms (Fig. 5 and data not shown). In addition, we analyzed the crystal structures of eEF1A from rabbit (PDB code 4C0S (7), *Aeropyrum pernix* (PDB codes 3WXM (22) and 3VMF (23), and *Sulfolobus acidocaldarius* (PDB codes 1SKQ (24) and 1JNY (6))). In
were viable and did not display growth defects under a variety of conditions, including temperature stress, osmotic stress, and endoplasmic reticulum stress in the presence of benomyl. The alignment was prepared with ClustalW with eEF1A from various organisms. The alignment was used to compare the initial velocities of glycosylation of non-glutaminylated (eEF1A purified from mouse liver) versus glutaminylated eEF1A (purified from S. cerevisiae) (supplemental Fig. 4). Additionally, we compared the glucosylation of the ternary complex (eEF1A, GTP, and Phe-tRNAPhe) constituting the bona fide substrate of Lgt3. We found that the initial glucosylation rate of glutaminylated and non-glutaminylated eEF1A within the ternary complex was similar, showing that Glu45 glutaminylation does not influence modification of the elongation factor at Ser53 by Lgt3.

**Discussion**

Here we show, by using tandem mass spectrometric analysis, site-directed mutagenesis, and structural data that eEF1A from yeast is modified by the attachment of a single glutamine moiety to amino acid Glu45 within the GTPase domain. Structural data reveal covalent linkage of i-glutamine via the α amino group.

Although glutaminylation and polyglutamylation (the attachment of glutamic acids) are well-known posttranslational modifications (e.g. modification of α- and β-tubulin (20)) catalyzed by γ-glutamyltransferase (21), the posttranslational modification of proteins by attachment of glutamine (glutaminylation) via the α amino group has not been reported previously.

The chemical principle of the novel posttranslational modification reaction reported here appears to be similar to the reaction catalyzed by glutaminyl-tRNA synthetase (Gln-RS) during the charging of tRNA at the 3' acceptor stem region, which results in the same covalent linkage of a glutaminyl moiety (25). Several aminocyl-tRNA synthetase (aa-RS) paralogs with unknown functions exist in various species, including yeast. The class II lysyl-tRNA synthetase paralog GenX/PoxA/YjeA was shown to attach a lysine residue to another lysine of /H9251/[H9252]/-tubulin (20) catalyzed by /H9253/[H9254]amino group has not been reported previously.

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site of glutaminylation in eEF1A is located close to the 3’ aminoacyl acceptor stem of the eEF1A-bound aa-tRNA (supplemental Fig. 5).

Figure 5. Mouse elongation factor 1A is not glutaminylated at Glu45. A, SDS-PAGE of eEF1A affinity-purified from mouse liver (mEF1A), which was used for mass spectrometric analysis. B, CID MS-MS revealed no modification of Glu45 in the chymotryptic peptide 43-EKEFAEMGKGSF-54 (precursor $m/z = 642.3 (2+)$. Immon., immonium ion; Seq., sequence. C, a representative LC/MS-MS extracted ion chromatogram of the peptide 43-EKEFAEMGKGSF-54 peak at $m/z = 642.3 (2+)$ from mouse eEF1A.

So far, it is unclear whether glutaminylation in yeast is a transient and reversible modification. Despite extensive efforts, we were not able to identify unmodified eEF1A in yeast cells. Anal-
ytes performed at different cell stages of yeast growth exclusively resulted in detection of completely glutaminylated eEF1A (supplemental Fig. 1). By contrast, we were not able to detect glutaminylmethylation of mammalian, fish, or insect eEF1A. The site of glutaminylmethylation and the nature of the acceptor amino acid is highly specific. We observed that amino acid point mutations of the glutaminyl attachment site (Glu45) of yeast eEF1A prevented glutaminylmethylation, even when glutamate was replaced by negatively charged aspartic acid with a C1 atom-shortened side chain. This demonstrates that the attachment of glutamine is strictly specific for Glu45.

Besides glutaminylmethylation of eEF1A, there are three other “unusual” posttranslational modifications also found in eukaryotic ribosome-associated factors (28): EPG modification of Glu301 and Glu374 in eEF1A, hypusine modification of eukaryotic initiation factor 5A (eIF5A), and diphthamide modification of eukaryotic elongation factor 2 (eEF2) (29). The precise functional roles of these modifications are not well-understood. Although the diphthamide modification might play a role in translation fidelity (30), the function of hypusine and EPG modifications are still enigmatic (28). Expression of a glutaminylated eEF1A mutant, as in the eEF1A E45K mutant, resulted in detection of completely glutaminylated eEF1A. Expression of an eEF1A mutant with a glutaminyl attachment site (Glu45) from S. cerevisiae, after deletion of endogenous eEF1A, demonstrated that attachment of glutamine to eEF1A is not essential for growth. Surprisingly, translation stress conditions induced by Geneticin, paromomycin, or anisomycin showed growth retardation of native glutaminylated eEF1A (local neutral charge) or eEF1A with an introduced bulkier side chain of lysine at position Glu45 (local positive charge) in comparison with the E45D mutant (local negative charge). Thus, in the eEF1A E45K mutant, as in the Glu45-glutaminylated eEF1A, the effect of the bulky and positively charged side chain might influence the interaction with the ribosomal factor-binding site and, therefore, translation efficiency. These data suggest that, in yeast, glutaminylmethylation might have a regulatory function in protein synthesis. Previously, we reported that Ser53 of eEF1A is modified by glucosylation catalyzed by the L. pneumophila effectors Lgt1–3 (15, 16). We found that Lgt-induced glucosylation did not differ with glutaminylated and non-glutaminylated eEF1A, which is a further indication that modification of Glu45 did not cause drastic structural changes of the molecule.

Recently, the mammalian elongation complex structure with eEF1A bound to the 80S ribosome has been reported (31). In this complex, the conserved Glu45 is oriented close to the sarcin–ricin loop. The sarcin–ricin loop is suggested to stimulate the GTPase activity of eEF1A after codon–anticodon recognition at the A-site of the ribosome (31, 32). Analysis of the described mammalian 80S ribosome complex reveals that glutaminylmethylation of amino acid Glu45 of eEF1A would be accommodated in this complex. On the other hand, it is conceivable that the glutaminylmethylation is involved in binding of the eEF1A ternary complex to the factor-binding site of the ribosome and, therefore, has the potential to influence translation (supplemental Fig. 5).

Taken together, here we describe a novel postranslational modification of yeast eEF1A by glutaminylmethylation. In all organisms, the genomic content is restricted to a specific protein repertoire. Fine-tuning of protein functions (e.g. in signaling events) and/or functional extensions are achieved by a large array of postranslational modifications, resulting in additional diversification of the proteome. Translation elongation factors especially appear to be substrates of an extended spectrum of postranslational modifications not observed in other proteins. Our findings add another type of postranslational modification to this spectrum. It remains to be clarified whether glutaminylmethylation is restricted to eEF1A or also observed in other proteins. Identification of the responsible enzyme will be an essential requirement for future detailed characterization of this novel postranslational modification.

Experimental procedures

Strains, vectors, and materials

E. coli DH10B and TG1 were used for cloning and E. coli BL21 (DE3) for recombinant protein production (Invitrogen). Haploid S. cerevisiae MH272–3fa (ura3, leu2, his3, trp1, ade2) or diploid MH272–3fa/a (ura3/ura3, leu2/leu2, his3/his3, trp1/trp1, ade2/ade2) was used for yeast genetic studies (33). The following commercial E. coli and S. cerevisiae/E. coli shuttle vectors were used: pET28a, pET11 (Novagen, Madison, WI), and pRS313 (His3) (34). For yeast transformation, standard genetic techniques were applied (35). All sequences of the corresponding plasmids and site-directed mutations were confirmed by sequencing (GATC Inc., Konstanz, Germany). DNA-modifying enzymes, Pfu DNA polymerase, 5-fluoroorotic acid, and kits for plasmid DNA and PCR product isolation were purchased from Fermentas (St. Leon-Rot, Germany). UDP-[14C]glucose was from American Radiolabeled Chemicals (St. Louis, MO). The components of liquid medium for S. cerevisiae and E. coli were from Difco (BD Biosciences) and Carl Roth GmbH (Karlsruhe, Germany), respectively. S. cerevisiae was cultivated in YPD (1% yeast extract, 2% peptone, and 2% glucose) or minimal (0.67% yeast nitrogen base without amino acids and with ammonium sulfate, 2% glucose or 2% galactose, and the corresponding supplement) medium at 30 °C. S. pombe was cultivated in YES medium (0.5% yeast extract, 3% glucose, and 0.02% of each) adenine, leucine, histidine, and uracil). Glucose, galactose, uracil, adenine, histidine, tryptophan, lysine, and leucine were from Sigma. The yeast DNA isolation kit was from Pierce (Thermo Fisher Scientific, Bonn, Germany). Isopropyl-β-D-thiogalactosidase was from Roth. RNase inhibitor was from Promega (Mannheim, Germany). All other reagents were of analytical grade and purchased from commercial sources.
Protein purification

The cloning, recombinant expression and purification of Lgt3 (gene lgt3/lpg1488) from L. pneumophila strain Philadelphia-1 was described previously (36). For recombinant eEF1B (37) and Lgt3 protein production, the corresponding genes were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at 22 °C overnight on a shaker. Bacterial cells were collected by centrifugation and lysed by French press on ice. The proteins were purified by nickel affinity chromatography using HisTrap columns (GE Healthcare) connected to an AKTA purifier (GE Healthcare) and stored in 10% glycerol/TBS solution at −80 °C. Purification of native EF1A from S. cerevisiae, S. pombe, H. volcanii, zebrafish ZF4 cells, bovine liver tissue, mouse brain or liver, or total G. mellonella homogenate was performed by a method based on eEF1A interaction with His-tagged eEF1Bα as described previously (37).

Cloning and mutagenesis of S. cerevisiae eEF1A

The coding sequence of yeast EF1A with ~500-nt upstream and downstream regions was amplified from S. cerevisiae chromosomal DNA using primers 5′-CATATCAGATGGACCCAAAGTTACTGGAAGCG and 5′-CATATCAGATGCGTCGAACCAGAGCTATT (engineered restriction endonuclease sites are underlined). The PCR product was digested with BamHI/SalI restriction endonucleases and ligated into similarly digested pRS313. Site-directed mutagenesis was performed as described previously using the QuickChange (Stratagene) technology with the oligonucleotides 5′-GTTGGAATTTGCAGCTGAATTAG/5′-CTAATTCAAGCGAGATCCTTTCGAAC, 5′-GTTGGAATTTGCAGCTGAATTAG/5′-CTAATTCAAGCGAGATCCTTTCGAAC and 5′-GTTGGAATTTGCAGCTGAATTAG/5′-CTAATTCAAGCGAGATCCTTTCGAAC to generate eEF1A with the E45D, E45K, and E45A substitutions, respectively.

Construction of S. cerevisiae strains with eEF1A mutations

Construction of S. cerevisiae variant with inactivated chromosomal copies of eEF1A and containing wild-type eEF1A on an Ura3 marker–containing plasmid was described previously (38). This strain was transformed individually with the pRS313-based plasmids coding for eEF1A wild-type, E45D, E45K, and E45A and passed over 5-fluoroorotic acid–containing agar plates (39) to remove the initial Ura3 marker–carrying plasmid.

Yeast growth assay

To estimate yeast growth phenotypes, engineered S. cerevisiae cells were titrated 10-fold from the starting value of A590 = 1.0. From each dilution, an aliquot of 5 μl of suspension was dropped onto SD agar supplemented with the corresponding marker substances. Where indicated, additional stress agents were included (20 mM DTT, 2% galactose, 50 ng/ml Geneticin, 350 μg/ml paromomycin, 5 μg/ml anisomycin, 1 M sorbitol, 1 M NaCl, 1 M KCl, 10 μg/ml benomyl, 0.15% caffeine, or 200 μg/ml polymyxin), or the pH level was adjusted correspondingly. Petri plates were incubated for 3–5 days at 30 °C (or at the temperature mentioned in the figure legends) before photography. In comparison with liquid culture experiments, assays on solid medium led to results that were more conclusive.

Glucosyltransferase assay

Glucosylation was performed with 140 nm recombinant Histagged Lgt3 and eEF1A or the eEF1A-GTP-Phe-aatRNAPeptide ternary complex (3 μM) in a total volume of 20 μl. Production of Phe-tRNAPeptide and ternary eEF1A complex formation was described as described previously (36). The standard glycosylation reaction was performed at 30 °C for the indicated times in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MnCl2, and 10 μM UDP-[14C]glucose. The reaction was stopped by addition of SDS sample buffer and heating at 95 °C for 5 min. Subsequently, the samples were subjected to polyacrylamide gel electrophoresis (40). Proteins were stained with Coomassie Brilliant Blue 250, and radiolabeled bands were analyzed by phosphorimaging and quantified using ImageQuant 5.2 (GE Healthcare) and Sigma Plot.

LC/MS-MS analysis

For in-gel digestion, the excised gel bands were destained with 30% acetonitrile, shrunk with 100% acetonitrile, and dried in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with trypsin were performed at 37 °C and digests with chymotrypsin at 25 °C overnight in 0.1 M NH4HCO3 (pH 8). About 0.1 μg of protease was used for one gel band. Peptides were extracted from the gel slices with 5% formic acid. LC/MS-MS analyses were performed on a Q-TOF (quadrupole time-of-flight) mass spectrometer (Agilent 6520, Agilent Technologies) and on an ion trap mass spectrometer (Agilent 6340, Agilent Technologies) equipped with an ETD source. Both instruments were coupled to a 1200 Agilent nanoflow system via a HPLC chip cube electrospray ionization interface. Peptides were separated on an HPLC chip with an analytical column of 75-μm inner diameter and 150-mm length and a 40-nl trap column, both packed with Zorbax 300SB C-18 (5-μm particle size). Peptides were eluted with a linear acetonitrile gradient with 1%/min at a flow rate of 300 nl/min (starting with 3% acetonitrile). The Q-TOF was operated in the 2-GHz extended dynamic range mode. MS-MS analyses were performed using data-dependent acquisition mode. After an MS scan (2 spectra/s), a maximum of three peptides were selected for MS-MS (2 spectra/s). Singly charged precursor ions were excluded from selection. Internal calibration was applied. The ETD ion trap was operated in data-dependent acquisition mode. After an MS scan (standard enhanced mode), a maximum of three peptides were selected for MS-MS (standard enhanced mode). The ICC control for the survey scan was set to 350,000, and the maximum accumulation time was set to 300 ms. The accumulation time for fluorophore was set to 4 ms (according to an ICC of 500,000–600,000). The ICC control for the survey scan was set to 350,000, and the maximum accumulation time was set to 300 ms. The accumulation time for fluorophore was set to 4 ms (according to an ICC of 500,000–600,000). The Q-TOF reaction was performed at 30 °C for the indicated times in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MnCl2, and 10 μM UDP-[14C]glucose. The reaction was stopped by addition of SDS sample buffer and heating at 95 °C for 5 min. Subsequently, the samples were subjected to polyacrylamide gel electrophoresis (40). Proteins were stained with Coomassie Brilliant Blue 250, and radiolabeled bands were analyzed by phosphorimaging and quantified using ImageQuant 5.2 (GE Healthcare) and Sigma Plot.
mass tolerance, 20 ppm (Q-TOF)/1.1 Da (ion trap); MS-MS mass tolerance, 0.05 Da (Q-TOF)/0.3 Da (ion trap); enzyme, "trypsin" or "chymotrypsin" with two uncleaved sites allowed; variable modifications, carbamidomethyl (C), Gln-> pyroGlu (N-term), Q, oxidation (M), and hexose (ST). For protein identification, a custom database was used.

X-ray structure analysis and refinement

The vicinity of Glu^{45} in the 1.67-Å-resolution X-ray structure of eEF1A from S. cerevisiae (PDB code 1F60 (8)) was inspected for unassigned electron density, which was calculated using TLS (translation, libration, screw motion) refinement in phenix.refine (41) with coordinates and structure factors retrieved from the Protein Data Bank. The ΔF_{obs}–ΔF_{calc} difference electron density map clearly indicated the position and orientation of the posttranslational modification. Water molecules in the proximity of Glu^{45} were removed, and the glutaminylation was manually inserted as covalent attachment to Glu^{45} in a peptide bond–like manner between the α amino group of glutamine and the δ C atom of the Glu^{45} side chain. The structure was refined employing restraints for a peptide-like bond according to Ref. 42. Specifically, refinement targets for atomic distances were set to 1.336 Å (N_{Glu}–C_{δ2α}), 1.229 Å (C_{δ2α}–O_{ε2α}), 1.459 Å (C_{δ2α}–N_{Glu}), and 1.525 Å (C_{ψ2α}–C_{γ2α}). Refinement targets for the angles were set as follows: 121.7° (C_{δ2α}–N_{Glu}–C_{ψ2α}), 122.7° (N_{Glu}–C_{δ2α}–O_{ε2α}), 120.1° (O_{ε2α}–C_{δ2α}–C_{γ2α}), and 117.2° (N_{Glu}–C_{δ2α}–C_{γ2α}). In addition, planarity restraints were used for atoms C_{δ2α}, N_{Glu}, C_{δ2α}, C_{ψ2α}, C_{γ2α} and O_{ε2α}. After refinement, these distances and the plane were identical to the ideal values. The quality of the model and fit to the electron density were compared for L- and D-glutamine, and the ideally fitting L-enantiomer was included in the final structure. Water molecules were inserted, and model building was completed using COOT (43). This included insertion of three polyethylene glycol moieties and two additional posttranslational modifications an Ne-monomethylation of Lys^{68} and Ne,Ne,Ne-trimethylation of Lys^{77}. Electron density was visible for an additional short α-helical peptide that was likely to be part of the C terminus of eEF1A, as fragmentary electron density at 1σ and continuous electron density at 0.5σ connected that peptide to the C terminus of eEF1A. However, the connecting loop was not ordered enough to allow refinement. The structure was refined using phenix.refine with TLS to a crystallographic R and R_{free} of 15.6% and 18.4%, respectively. The quality of the structure was checked using MolProbity (44). There was no Ramachandran outlier, and 97.44% of the residues were in the favored regions. Data refinement statistics are summarized in supplemental Table 1. The figures were prepared using the PyMOL molecular graphics system (version 1.5.0.4, Schrödinger, LLC).

Author contributions—T. J. designed and conducted experiments, analyzed the data, and wrote the paper. A. S., Z. H., and J. D. performed mass spectrometric analyses. C. W., C. H., and G. R. A. conducted structural analyses. Y. B. and S. R. performed yeast experiments. T. T. performed glycosylation experiments. K. A. designed the study, analyzed the data, and wrote the paper. All authors discussed the results and commented on the manuscript.

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References

1. Andersen, G. R., Nissen, P., and Nyborg, J. (2003) Elongation factors in protein biosynthesis. Trends Biochem. Sci. 28, 434–441
2. Khachoo, M., Mehdiali, K., Pilon-Larose, K., Pause, A., Côté, J., and Lee, S. (2008) eEF1A is a novel component of the mammalian nuclear export machinery. Mol. Biol. Cell 19, 5296–5308
3. Panasyuk, G., Nemazany, I., Filonenko, V., Negrutskii, B., and El'skaya, A. V. (2008) A2 isoform of mammalian translation factor eEF1A displays increased tyrosine phosphorylation and ability to interact with different signalling molecules. Int. J. Biochem. Cell Biol. 40, 63–71
4. Mateyak, M. K., and Kinzy, T. G. (2010) eEF1A: thinking outside the ribosome. J. Biochem. Mol. Biol. 43, 21209–21213
5. Lambert, A., Caraglia, M., Longo, O., Marra, M., Abbruzzese, A., and Arcari, P. (2004) The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. Amino Acids 26, 443–448
6. Vitagliano, L., Masullo, M., Sica, F., Zagari, A., and Bocchini, V. (2001) The crystal structure of Sulfolobus solfataricus elongation factor 1α in complex with GDP reveals novel features in nucleotide binding and exchange. EMBO J. 20, 5305–5311
7. Crepin, T., Shalak, V. F., Yaremchuk, A. D., Vlasenko, D. O., McCarthy, A., Negrutski, B. S., Tukalo, M. A., and El’skaya, A. V. (2014) Mammalian translation elongation factor eEF1A2: X-ray structure and new features of GDP/GTP exchange mechanism in higher eukaryotes. Nucleic Acids Res. 42, 12939–12948
8. Andersen, G. R., Pedersen, L., Valente, L., Chatterjee, I., Kinzy, T. G., Kjeldgaard, M., and Nyborg, J. (2000) Structural basis for nucleotide exchange and competition with tRNA in the yeast elongation factor complex eEF1A:eEF1B. Mol. Cell 6, 1261–1266
9. Gross, S. R., and Kinzy, T. G. (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat. Struct. Mol. Biol. 12, 772–778
10. Gross, S. R., and Kinzy, T. G. (2007) Improper organization of the actin cytoskeleton affects protein synthesis at initiation. Mol. Cell. Biol. 27, 1974–1989
11. Cavallius, J., Zoll, W., Chakraburtty, K., and Merrick, W. C. (1993) Characterization of yeast EF-1α: non-conservation of post-translational modifications. Biochem. Biophys. Acta 1163, 75–80
12. Traug, J. A. (2001) Insulin, phorbol ester and serum regulate the elongation phase of protein synthesis. Prog. Mol. Subcell. Biol. 26, 33–48
13. Zobel-Thropp, P., Yang, M. C., Machado, L., and Clarke, S. (2000) A novel post-translational modification of yeast elongation factor 1A: methylation at the C terminus. J. Biol. Chem. 275, 37150–37158
14. Whiteheart, S. W., Shenbagamurthi, P., Chen, L., Cotter, R. J., and Hart, G. W. (1989) Murine elongation factor 1α (EF-1α) is posttranslationally modified by novel amide-linked ethanoamine-phosphoglycero moieties: addition of ethanoamine-phosphoglycerol to specific glutamic acid residues on EF-1α. J. Biol. Chem. 264, 14334–14341
15. Belyi, Y., Niggegweg, R., Optiz, B., Vogelgesang, M., Hinnenstiel, S., Wilim, M., and Aktories, K. (2006) Legionella pneumophila glucosyltransferase inhibits host elongation factor 1A. Proc. Natl. Acad. Sci. U.S.A. 103, 16953–16958
16. Belyi, Y., Tabakova, I., Stahl, M., and Aktories, K. (2008) Lgt: a family of cytoxic glucosyltransferases produced by Legionella pneumophila. J. Bacteriol. 190, 3026–3035
17. Eijji, S. (2002) Moonlighting functions of polypeptide elongation factor 1 from actin bundling to zinc finger protein R1-associated nuclear localization. Biocsi. Biotechnol. Biochem. 66, 1–21
SUPPLEMENTAL DATA

Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A

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Table S1
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Supplemental References
**Table S1**: Refinement statistics of the eEF1A-eEF1Bα complex.

|                       |                        |                |
|-----------------------|------------------------|----------------|
| Resolution (Å)        | 20.0 – 1.67            |                |
| Reflections           |                        |                |
| Refinement            | 63528                  |                |
| Test set              | 3217                   |                |
| Number of atoms       |                        |                |
| Protein (including PTM) | 4271                  |                |
| Water                 | 725                    |                |
| Ligands               | 30                     |                |
| R / R<sub>free</sub>  | 15.58 / 18.44          |                |
| R.m.s deviations      |                        |                |
| Bonds (Å)             | 0.009                  |                |
| Angles (°)            | 0.97                   |                |
| Ramachandran plot (%) |                        |                |
| Favored               | 97.44                  |                |
| Allowed               | 2.56                   |                |
| Disallowed            | 0.00                   |                |

Note: Data were collected and processed by Andersen *et al.* (1) and Pedersen *et al.* (2).
Glutaminylation of yeast eEF1A

Suppl. Fig. 1. Glutaminylation of yeast eEF1A is not depending on a specific growth phase. Extracted ion chromatogram of the chymotryptic peptide 43-EKEAAELGKGSFKY-56 of eEF1A (precursor m/z = 421.97 (4+)) isolated from *S. cerevisiae* grown in logarithmic (A) or stationary phase (B). The MS/MS data revealed quantitative modification of E45. The unmodified peptide could not be detected. Collision-induced dissociation MS/MS analysis of the tryptic peptide 42-FEKEAAELGK-51 of yeast eEF1A (precursor m/z = 642.3 (2+)) in logarithmic (C) or stationary growth phase (D) is shown.
Suppl. Fig. 2. Mono- and trimethylation sites in the eEF1A structure (PDB 5O8W). \( \text{N}^\varepsilon \)-monomethylated K30 (A) and \( \text{N}^\varepsilon,\text{N}^\varepsilon,\text{N}^\varepsilon \)-trimethylated K79 (B) of the eEF1A structure are shown in stick-and-ball presentation with surrounding 2mFo-dFc electron density map (grey) contoured at a level of 1.0 \( \sigma \).
Suppl. Fig. 3. *S. cerevisiae* expressing eEF1A E45 mutants were not influenced by various stress conditions. (A) Amount of engineered eEF1A in different variants of *S. cerevisiae*. Yeast were grown in YPD broth to an OD$_{600}$ = 0.5, lysed and tested in Western blotting with anti-eEF1A serum and with the sera against ribosomal proteins Rps9/Rpl24 as loading control. Please note that the concentration of eEF1A E45A is slightly reduced probably due to its instability or lower production. (B) Growth phenotypes of *S. cerevisiae* strains containing eEF1A with E45 substitutions. Serial dilutions of the corresponding yeast variants were spotted onto YPD agar supplemented with different stress agents (1 M sorbitol, 1 M NaCl, 1 M KCl, 10 μg/ml benomyl, 0.15% caffeine, 200 μg/ml polymyxin, 20 mM DTT, or pH 8) and cultivated for 72 h at 30°C or 15°C.
Suppl. Fig. 4. Glucosylation of S53 of eEF1A in the ternary complex is not affected by glutaminylation. Kinetics of $^{14}$C-glucosylation of non-glutaminylated eEF1A (A) and glutaminylated eEF1A (B) by *Legionella* glucosyltransferase Lgt3 in the absence (open circles) or presence of Phe-tRNA$^{Phe}$ and GTP (filled circles). The amount of eEF1A glucosylation is shown as the mean (+/- SD) of at least three independent experiments.
Suppl. Fig. 5. Location of E45 in rabbit eEF1A bound to the eukaryotic ribosome. Cryo-electron microscopy structure of the (80S) mammalian elongation complex comprising the large (60S, violet) and the small (40S, yellow) ribosomal subunits (PDB 5LZS) (3). tRNAs at the P and E site are shown in grey, mRNA is shown in dark red. Insert: The structure of eEF1A at the A-site of the ribosome is shown with E45 (red, stick-and-ball representation) located between the tRNA (orange) and the ribosomal L23 protein (blue). The sarcin-ricin loop is shown in violet.
SUPPLEMENTAL REFERENCES:

1. Andersen, G. R., Pedersen, L., Valente, L., Chatterjee, I., Kinzy, T. G., Kjeldgaard, M., and Nyborg, J. (2000) Structural basis for nucleotide exchange and competition with tRNA in the yeast elongation factor complex eEF1A:eEF1Balpha. *Mol. Cell* **6**, 1261-1266

2. Pedersen, L., Andersen, G. R., Knudsen, C. R., Kinzy, T. G., and Nyborg, J. (2001) Crystallization of the yeast elongation factor complex eEF1A-eEF1B alpha. *Acta Crystallogr. D. Biol. Crystallogr* **57**, 159-161

3. Shao, S., Murray, J., Brown, A., Taunton, J., Ramakrishnan, V., and Hegde, R. S. (2016) Decoding mammalian ribosome-mRNA states by translational GTPase complexes. *Cell* **167**, 1229-1240 e1215
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