

A Novel Post-translational Modification of Yeast Elongation Factor 1A

METHYLESTERIFICATION AT THE C TERMINUS*

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Protein methylation reactions can play important roles in cell physiology. After labeling intact *Saccharomyces cerevisiae* cells with S-adenosyl-L-[methyl-³H]methionine, we identified a major methylated 49-kDa polypeptide containing [³H]methyl groups in two distinct types of linkages. Peptide sequence analysis of the purified methylated protein revealed that it is eukaryotic elongation factor 1A (eEF1A, formerly EF-1α), the protein that forms a complex with GTP and aminoacyl-tRNAs for binding to the ribosomal A site during protein translation. Previous studies have shown that eEF1A is methylated on several internal lysine residues to give mono-, di-, and tri-N-ε-methyl-lysine derivatives. We confirm this finding but also detect methylation that is released as volatile methyl groups after base hydrolysis, characteristic of ester linkages. In cycloheximide-treated cells, methyl esterified eEF1A was detected largely in the ribosome and polysome fractions; little or no methylated protein was found in the soluble fraction. Because the base-labile, volatile [methyl-³H]radioactivity of eEF1A could be released by trypsin treatment but not by carboxypeptidase Y or chymotrypsin treatment, we suggest that the methyl ester is present on the α-carboxyl group of its C-terminal lysine residue. From the results of pulse-chase experiments using radiolabeled intact yeast cells, we find that the N-methylated lysine residues of eEF1A are stable over 4 h, whereas the eEF1A carboxyl methyl ester has a half-life of less than 10 min. The rapid turnover of the methyl ester suggests that the methylation/demethylation of eEF1A at the C-terminal carboxyl group may represent a novel mode of regulation of the activity of this protein in yeast.

Reversible covalent modification of proteins is a common mode of regulation in cell metabolism (1–3). A large number of protein phosphorylation and dephosphorylation reactions are involved in a variety of cell signaling and metabolic control reactions where specific kinases phosphorylate proteins using the γ-phosphate group of ATP and dephosphorylation of these

proteins occurs through the action of phosphatases (4). In a much smaller number of cases, methylation and demethylation reactions are also involved in cell signaling and, potentially, metabolic regulation (5). Methyltransferases use the methyl donor group on S-adenosylmethionine (AdoMet)¹ to methylate various substrates; methylesterases act to demethylate them.

The yeast *Saccharomyces cerevisiae* contains the *STE14* isoenzyme of prenylcysteine methyltransferase that has been shown to methylate a wide range of proteins within the cell (6, 7). Its known substrates include the small G-proteins RAS1 and RAS2 and the secreted peptide a-factor (8–10). The specific role of this methyltransferase is unknown, but it has been suggested that methylation of its products increases their hydrophobicity to help direct them to the membrane, decreases their rate of proteolytic degradation, and may modulate their protein-protein interactions involved in cell signaling (6).

In an attempt to find new reversible AdoMet-dependent methylation/demethylation reactions in yeast, we constructed a mutant strain (*MY101*) lacking the gene that encodes the *STE14* methyltransferase and also the two genes encoding the AdoMet synthetases (*SAM1* and *SAM2*). Yeast cells contain a specific permease in the outer membrane that actively takes up AdoMet from the surrounding medium (11). Because of its AdoMet synthetase defect, *MY101* depends on exogenous AdoMet for growth. We thus supplied isotopically labeled [methyl-³H]AdoMet during log phase growth and analyzed *in vivo* radiolabeled proteins.

We found a major methylated 49-kDa species that contains both amino-methylated and carboxyl-methylated linkages. We purified the methylated 49-kDa protein from a radiolabeled yeast cytosolic extract and performed N-terminal sequence analysis of its tryptic peptides. This work revealed that the 49-kDa polypeptide is elongation factor 1A (eEF1A), which leads aminoacyl-tRNAs to their codon-dependent placement in the A site of the ribosome (12). eEF1A has already been purified and characterized from *S. cerevisiae* (13–15). It is known to contain internal amino-methylated lysine residues (16) with unknown function(s) (17). However, the existence of the C-terminal carboxyl methyl ester identified in this study has not been previously recognized. The rapid turnover of the methyl ester of eEF1A in intact cells indicates that its function may be reversibly regulated by this modification.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—*S. cerevisiae* strain W744-1A (*MATa his3 leu2 ade2 trp1 ura3 sam1::LEU2 sam2::HIS3*)

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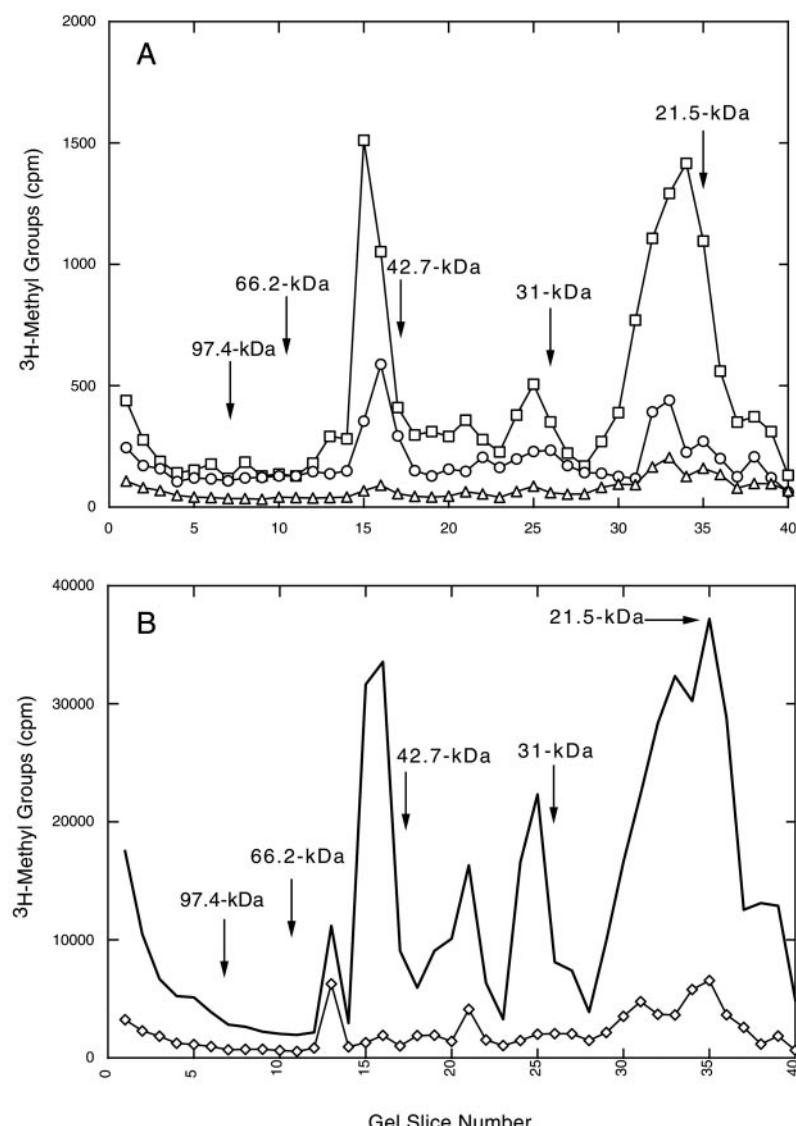
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¹ The abbreviations used are: AdoMet, S-adenosyl-L-methionine; eEF1A, eukaryotic elongation factor 1A (formerly EF-1α); [³H]AdoMet, S-adenosyl-L-[methyl-³H]methionine; MES, 2-(N-morpholino)ethanesulfonic acid; CPY, carboxypeptidase Y.

FIG. 1. Evidence for an abundant methyl esterified 49-kDa polypeptide in yeast. *S. cerevisiae* strain MY101 was grown and labeled *in vivo* with [³H]AdoMet as described under “Experimental Procedures.” Cells were lysed, and polypeptides (~250 µg of protein/lane) were separated by 10% polyacrylamide-SDS gel electrophoresis. Gel slices were analyzed for [³H]methyl esters using H₂O (triangles), 0.2 M HCl (circles), 1.5 M Na₂CO₃ (squares) (A), and 2 M NaOH (diamonds) (B) to hydrolyze the methyl ester linkage (see “Experimental Procedures”). The total radioactivity (solid line, B) was determined after digestion of gel slices with 30% H₂O₂, as described under “Experimental Procedures.” The positions of the molecular mass markers electrophoresed in parallel lanes are indicated with arrows. These markers include rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa).



was provided by Dr. Yolande Surdin-Kerjan at the Center de Génétique Moléculaire (Gif-sur-Yvette, France). Strain MY101 (*ste14::URA3*, otherwise isogenic to W744-1A) was constructed by transforming strain W744-1A with the *Cla*I-BamHI fragment of plasmid pSM284 (a gift from Dr. Susan Michaelis at Johns Hopkins University) that contains the *STE14* gene with a portion of its coding region replaced by the *URA3* coding sequence (10). *URA*⁺ transformants were selected and tested to show the *ste14*-defective mating phenotype. Cells were grown at 30 °C in YPD medium (1% (w/v) yeast extract (Difco Laboratories), 2% (w/v) bacto-peptone (Difco), and 2% (w/v) D-glucose) supplemented with a final concentration of 100 µM *S*-adenosyl-L-methionine HSO₄⁻ salt (AdoMet) from Roche Molecular Biochemicals.

Isotopic Labeling—Strain MY101 was grown to early log phase ($A_{600\text{ nm}}$ between 0.6 and 0.8). Generally, an aliquot of 5 $A_{600\text{ nm}}$ units (5×10^8 cells) was harvested by centrifugation at $1000 \times g$ for 3 min at room temperature. Cells were washed three times with 1 ml of YPD medium. The final pellet (in a 1.5-ml polypropylene microcentrifuge tube) was resuspended in YPD containing *S*-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet) (892 µl of YPD + 108 µl [³H]AdoMet from Amersham Pharmacia Biotech, 77 Ci/mmol, 1 mCi/ml in dilute HCl (pH 2–2.5), ethanol (9:1 v/v)) to give a final concentration of [³H]AdoMet of 1.4 µM. Cells were incubated in a gyratory shaker at 225 rpm for 30 min at 30 °C, then pelleted at $13,600 \times g$ at room temperature for 1 min, and finally washed twice with 1 ml of water. In cases where more cells were labeled, the volume of reagents were adjusted proportionally.

Preparation of Cell Extracts—Isotopically labeled cells prepared as described above were resuspended in 50 µl of a lysis solution containing 1% SDS (w/v) and 0.67 mM phenylmethylsulfonyl fluoride; the latter was prepared from a 100 mM stock in Me₂SO. For column chromatography experiments, the lysis solution contained 20 mM MES-Tris, pH

6.5, and a protease inhibitor mixture (described below) instead of SDS. Glass beads (0.2 g, acid-washed, and 0.5-mm diameter from Biospec Products, Inc.) were then added to the cell suspension, and the tube was vortexed for 1 min followed by an incubation on ice for 1 min. This cycle was repeated seven times. Approximately 40 µl of this extract (300–400 µg of total protein) was removed with a micropipet tip; this fraction was used as the “cell lysate.”

Nonisotopically labeled cell extracts were prepared using French pressure cell lysis. Cell cultures (1 liter) were grown at 30 °C to an $A_{600\text{ nm}}$ of 2.0. Cells were harvested at $4,400 \times g$ for 7 min at 4 °C and washed with 20 ml of 50 mM Tris-Cl, pH 7.5. The resulting cell pellet (5.5 g of wet weight) was resuspended in 11 ml of the same buffer. A mixture of protease inhibitors in Me₂SO was added to give final concentrations of 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin. The cell suspension was passed twice through a French pressure cell at 20,000 p.s.i., and the resulting lysate was spun at $23,000 \times g$ for 30 min at 4 °C. The supernatant from this centrifugation was utilized for purification of the 49-kDa polypeptide.

SDS Gel Electrophoresis and Analysis of [³H]Methylated Polypeptides—Unless otherwise described, samples were resuspended in an equal volume of sample buffer (35.5% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, 6% (w/v) SDS, 0.18 M Tris-Cl, pH 6.8, 0.005% (w/v) bromophenol blue), heated at 100 °C for 5 min, and loaded onto 1.5- or 0.8-mm-thick slab gels containing a stacking gel and a 10.5-cm resolving gel in the Tris/glycine buffer system (18). The resolving gel was made from 10 or 12% (w/v) acrylamide and 0.28 or 0.43% (w/v) *N,N*-methylenebisacrylamide, respectively. Molecular mass standards (Bio-Rad number 161-0304) were used at 2 µg of protein each. Samples were electrophoresed with a constant current (35 mA for thick gels and 5–10

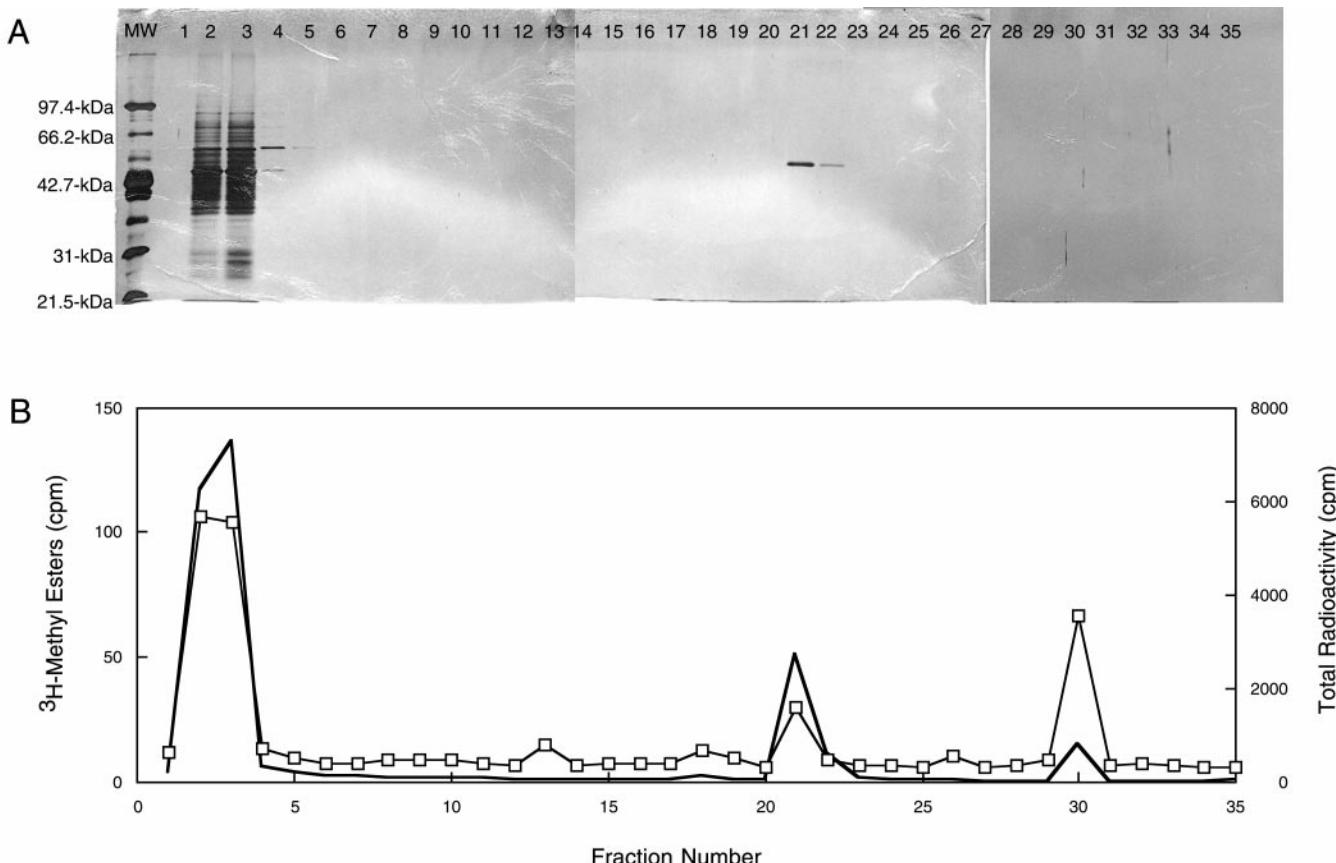


FIG. 2. Purification of the 49-kDa polypeptide using carboxymethyl cellulose cation exchange chromatography. *S. cerevisiae* strain MY101 was grown at 30 °C and 20 $A_{600\text{nm}}$ units of cells were labeled *in vivo* with 1.4 μM [³H]AdoMet for 30 min at 30 °C as described under “Experimental Procedures.” The cell lysate was diluted to 1 ml with 20 mM MES-Tris, pH 6.5, and was digested for 30 min with ribonuclease-A (100 μg , bovine pancreas, Sigma, boiled at 100 °C for 15 min). The mixture was then centrifuged at 100,000 $\times g$ for 1 h at 4 °C. The supernatant (900 μl) was removed and mixed with an aliquot (500 μl , 5 mg protein) of nonisotopically labeled cell extract. This mixture was applied to a 15-ml carboxymethyl cellulose column (CM52 from Whatman) equilibrated with 20 mM MES-Tris, pH 6.5, and 25% glycerol buffer at room temperature. The column was washed with 30 ml of the same buffer and then eluted by gravity flow with a step gradient of 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, and 1 M NaCl in the same buffer (25 ml each step). Fractions (3 ml) were collected, and aliquots (1.5 ml) were concentrated by adding 1.5 ml of 25% trichloroacetic acid (w/v), incubating at room temperature for 20 min, and then pelleting the protein precipitate for 25 min at 13,600 $\times g$. The pellets were washed with 100 μl of acetone (−20 °C), which was removed after an additional spin, and resuspended in 55 μl of the electrophoresis sample buffer. *A*, silver-stained polypeptides in each fraction analyzed for [³H]methyl esters using 1.5 M Na_2CO_3 (squares) and total radioactivity using 30% H_2O_2 (solid line).

mA for thin gels) until the dye front ran off the end of the resolving gel. Gels were stained for 15 min with 0.1% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid in water, destained overnight in 5% methanol and 10% acetic acid in water, washed for 10 min in 5% glycerol, and then vacuum-dried at 65 °C for 70 min onto Whatman 3MM chromatography paper.

Dried gel lanes 0.8 cm wide were cut into 0.3-cm slices unless otherwise indicated. To release [³H]methyl esters from polypeptides, 150 μl of 1.5 M Na_2CO_3 (pH measured at 12) was added to each gel slice in a 1.5-ml polypropylene microcentrifuge tube. In control experiments, 150 μl of 2 M NaOH, 0.2 M HCl, or water was added instead of the 1.5 M Na_2CO_3 . These uncapped tubes were carefully placed into 20-ml plastic scintillation vials containing 5 ml of Safety Solve counting fluid (Research Products International, Mt. Prospect, IL), so that no counting fluid entered the microcentrifuge tube. The vials were then capped tightly, incubated for 24 h at 37 °C, and then counted in a Beckman LS6500 scintillation counter. Here, [³H]methanol resulting from base-catalyzed hydrolysis of protein [³H]methyl esters diffuses from the microcentrifuge tube through the vapor phase and accumulates in the counting fluid. To determine the total radioactivity present in each gel slice, 1 ml of 30% H_2O_2 was gently added to each microcentrifuge tube within the vials after they were counted for [³H]methanol. Vials were capped loosely and incubated at 37 °C for an additional 24 h. Vials were then tightly capped, shaken vigorously to mix the contents of the vial, and counted.

Elution of the [³H]Methylated 49-kDa Polypeptide from a Polyacrylamide Gel Matrix—Polypeptides from *in vivo* [³H]AdoMet-labeled yeast were separated by 10% acrylamide SDS gel electrophoresis as

described above. The 49-kDa region was identified in an unstained portion of the wet gel relative to an adjacent lane containing molecular mass markers whose positions were identified by Coomassie staining. This region was cut into 0.3-cm slices, and each slice was placed into a 13 × 100-mm tube containing 1 ml of 50 mM Tris-Cl, pH 7.5. Each slice was finely chopped with the tip of a narrow spatula, and the tube was then capped and placed on an orbital shaker at 100 rpm at room temperature for about 18 h. An aliquot (50 μl) of the eluted polypeptides was counted for radioactivity to determine which tube(s) contained the [³H]methylated 49-kDa polypeptide.

Acid Hydrolysis of Polypeptides and Thin Layer Chromatography—The eluted 49-kDa polypeptide (25 μl) was mixed with 25 μl of 25% (w/v) trichloroacetic acid in a 6 × 50-mm glass vial, incubated at room temperature for 20 min, and then spun at 4000 $\times g$ for 25 min at room temperature. The pellet was washed once with acetone (100 μl , −20 °C), dried, and then acid-hydrolyzed in 6 N HCl at 110 °C for 20 h in a Waters Pico-Tag vapor-phase apparatus. For thin layer chromatography, the hydrolysate was mixed with unlabeled *N*- ϵ -monomethyl-lysine, *N*- ϵ -dimethyl-lysine, and *N*- ϵ -trimethyl-lysine and chromatographed on a 20-cm silica plate in methanol, 28% ammonium hydroxide (3:1). The position of the standards were determined from ninhydrin staining; the lane was then cut into 1-cm slices for quantitation of the radioactivity.

In-gel Assay for Sensitivity of Methyl Esters to Proteolytic Enzymes—Slices from a 0.8-mm-thick dried 12% acrylamide gel in the 49-kDa region were mixed with either 250 μl of 1 $\mu\text{g}/\text{ml}$ trypsin (Sigma, Type XI, bovine pancreas), 1 $\mu\text{g}/\text{ml}$ chymotrypsin (Sigma, Type II, bovine pancreas), or 50 $\mu\text{g}/\text{ml}$ carboxypeptidase Y (CPY) (Sigma, *S. cerevisiae*) each in a solution of 0.2 M Tris, 0.1 M citrate, pH 6, and 1% Triton X-100.

In control experiments, 250 μ l of 1.5 M Na₂CO₃ was used. The reactions proceeded in uncapped tubes placed carefully in scintillation vials containing 5 ml of counting fluid. The vials were capped tightly and incubated for 24 h at 37 °C (trypsin and 1.5 M Na₂CO₃) or 30 °C (chymotrypsin and CPY). [³H]Methyl esters and total radioactivity were analyzed as described above.

RESULTS

Characterization of a Major Methyl Esterified 49-kDa Polypeptide in *S. cerevisiae*—Yeast contain numerous methyl esterified polypeptides, many of which are formed by the action of the STE14 AdoMet-dependent C-terminal isoprenylcysteine methyltransferase (7). To identify novel methyl esterified proteins in yeast that may represent new types of reversible covalent modification, we utilized a *ste14* mutant strain where the gene for the isoprenylcysteine methyltransferase is disrupted. Yeast cells are able to take up AdoMet from the medium (11); this allows yeast methyl-accepting species to be efficiently radiolabeled *in vivo* using [*methyl*-³H]AdoMet in the culture medium. The *ste14* strain utilized in this study (MY101) also contains mutations in the genes encoding AdoMet synthetases *SAM1* and *SAM2*. These mutations make the cells dependent upon exogenously added AdoMet for growth and reduce the isotopic dilution of exogenous radiolabeled AdoMet by endogenously synthesized material within the cell.

S. cerevisiae strain MY101 was thus labeled *in vivo* with [*methyl*-³H]AdoMet to identify non-STE14-dependent methyl-accepting proteins as described under "Experimental Procedures." Cellular lysates were analyzed for the presence of polypeptide-bound [³H]methyl esters after fractionation by SDS gel electrophoresis. Here, gel slices were treated with mild base (1.5 M Na₂CO₃) to release volatile [³H]methanol, which can be trapped in scintillation fluid. Fig. 1A shows that a major 49-kDa methyl esterified polypeptide exists in these cells. Mild acid treatment (0.2 M HCl) of the 49-kDa species also releases volatile radioactivity as [³H]methanol, although in smaller amounts than the mild base treatment (Fig. 1A). However, when the gel slice was simply incubated in water, little or no volatile radioactivity was released from the 49-kDa polypeptide (Fig. 1A).

When the gel slices of the fractionated polypeptides were treated with a stronger base (2 M NaOH), volatile [³H]methylamine (from methylated arginine residues) was released from several polypeptides (Fig. 1B and Ref. 20) in addition to [³H]methanol from methyl esters. However, a similar amount of radioactivity was released at the 49-kDa position with either 2 M NaOH or 1.5 M Na₂CO₃, suggesting that methylated arginine residues are not present in this material.

We also determined the total amount of radioactivity in each of the gel slices (Fig. 1B). Interestingly, the amount of [³H]methanol released from the 49-kDa polypeptide was only about 5% of the total radioactivity found in the region of the 49-kDa polypeptide (Fig. 1, compare A and B). This result indicates that the polypeptide(s) in the 49-kDa region contain at least two types of methyl groups: one is in a base-labile methyl ester linkage (seen with the release of [³H]methanol in Fig. 1A), and one is in a base-stable linkage, which would be expected for methylamine derivatives such as methyl lysine or methyl histidine.

The amount of [³H]methyl esters incorporated into the 49-kDa polypeptide was found to be similar when cells were incubated with 2% glycerol in place of 2% D-glucose in the YPD medium, or when cells were labeled in a synthetic medium in the absence of a major carbon source (0.5% ammonium sulfate, 0.17% yeast nitrogen base (lacking amino acids), 0.008% adenine, and 0.0008% tryptophan). These results suggest that the protein methyl ester formation occurs during fermentative and

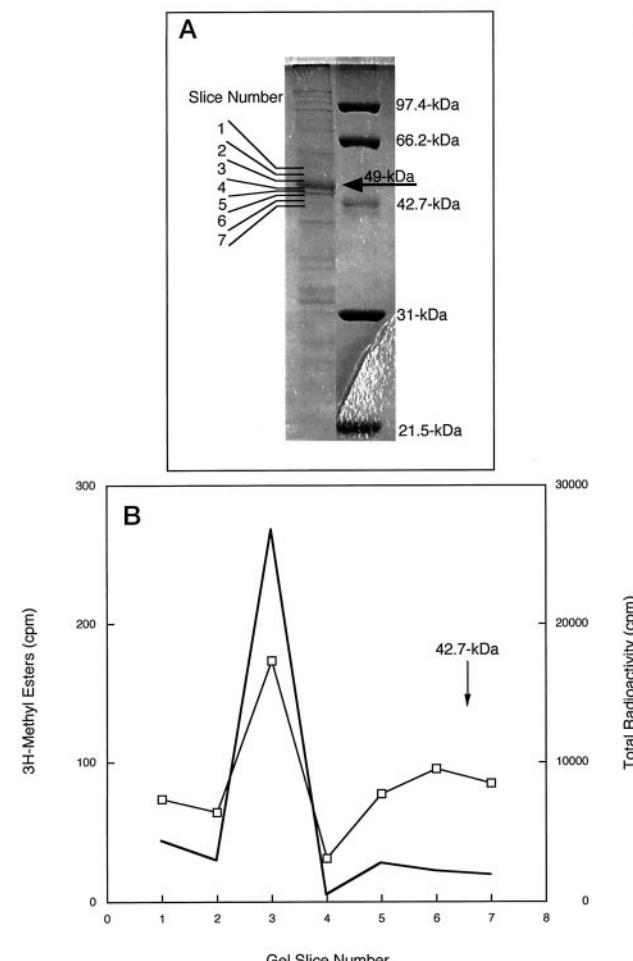


Fig. 3. Ribosome-bound eEF1A is methyl esterified. *S. cerevisiae* strain MY101 was labeled with [³H]AdoMet as in Fig. 1. Ribosomes were then isolated by a protocol based on that described by Savitz and Meyer (19). Cells were washed twice with H₂O and lysed in low salt buffer (50 mM Tris-Cl, pH 7.5, 25 mM potassium acetate, 5 mM magnesium acetate, and 0.67 mM phenylmethylsulfonyl fluoride) with glass beads. The cell lysate was diluted to 10 ml with low salt buffer and placed on a 15-ml cushion of 2.1 M sucrose in low salt buffer. The mixture was centrifuged at 110,000 \times g for 24 h at 4 °C. The pellet containing intact ribosomes was resuspended in 1.5 ml of high salt buffer (50 mM Tris-Cl, pH 7.5, 950 mM potassium acetate, and 5 mM magnesium acetate) and transferred to a 2-ml cushion of 1.8 M sucrose in high salt buffer and centrifuged at 110,000 \times g for 24 h at 4 °C. The pellet, containing ribosome-bound proteins, was resuspended in 500 μ l of LSB containing 0.25 M sucrose, and 30 μ l was analyzed by 12% polyacrylamide SDS gel electrophoresis (A). Dried gel slices in the 49-kDa region were cut as illustrated and analyzed for [³H]methyl esters (B, squares); total radioactivity (B, solid line) was determined using 30% H₂O₂, as described under "Experimental Procedures." Here, the major 49-kDa polypeptide (eEF1A) is contained entirely within slice 3; slice 4 contains the clear area below this polypeptide, slice 5 contains a minor polypeptide of about 46-kDa, and the other slices represent flanking regions.

nonfermentative growth conditions, as well as under non-growth conditions.

We have not attempted to characterize the radioactive peaks shown in Fig. 1 aside from the 49-kDa component. However, the broad 24-kDa peak appears to represent mostly methyl esters linked to RNA, because it largely disappears upon treatment with RNase (7). The smaller methyl ester peaks in the range of 32–40-kDa may be minor methylated species or degradation products of the 49-kDa component.

Analysis of the Base-stable Methyl Linkage(s) on the 49-kDa Polypeptide—*In vivo* [³H]AdoMet-labeled 49-kDa polypeptide from MY101 cells was eluted from the SDS gel matrix and was

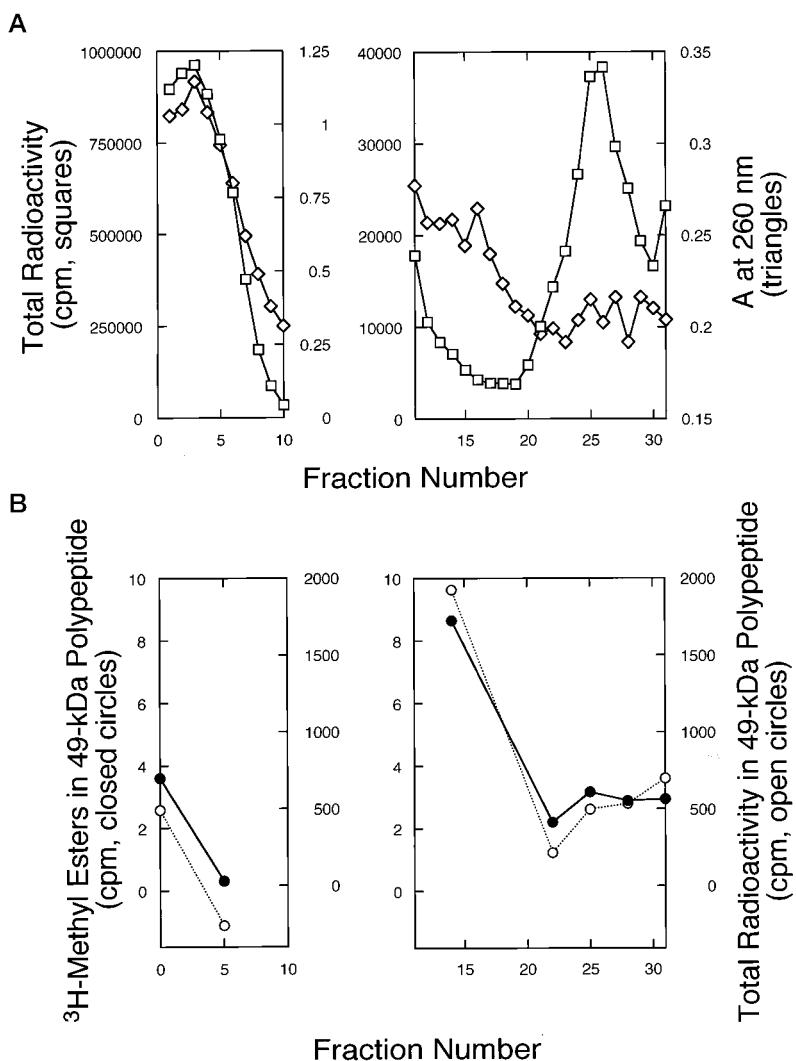


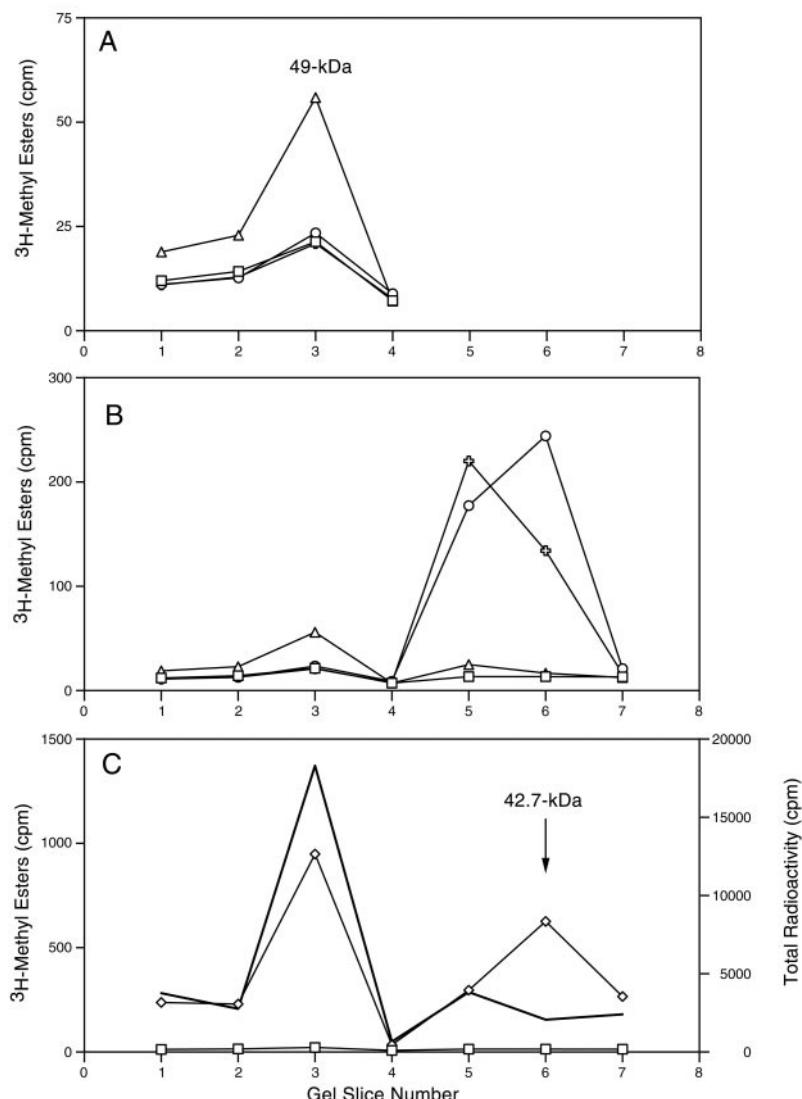
FIG. 4. Methyl esterified eEF1A fractionates with ribosomes and polysomes in cycloheximide-treated yeast cells. *S. cerevisiae* strain MY101 (50 $A_{600\text{nm}}$ units) was isotopically labeled with [³H]AdoMet as described under “Experimental Procedures” but in the presence of 0.1 mg/ml cycloheximide (Sigma). Polysomes and ribosomes were then separated from soluble proteins based on the protocol of Pintard *et al.* (25). Cells were pelleted and washed twice in breaking buffer (100 mM KCl, 20 mM sodium HEPES, pH 7.4, 2 mM magnesium acetate, 14.8 mM β -mercaptoethanol, and 0.1 mg/ml cycloheximide). Cells were resuspended to a final volume of 2 ml in this solution, and 2 g of glass beads were added. The mixture was frozen in liquid nitrogen, thawed at room temperature, and vortexed for 45 s. The resulting cell lysate was clarified by centrifugation at 14,000 \times *g* for 10 min. A portion of the supernatant was saved for analysis, and the remaining 1.8 ml was applied to a 0–50% linear sucrose gradient in breaking buffer. The gradient was centrifuged in a Beckman SW41 swinging bucket rotor at 275,000 \times *g* for 2 h at 4 °C. Fractions (375 μ l) were collected from the top of the gradient as a dense (1.85 g/cm^3) fluorinert solution was pumped in from the bottom. *A*, total radioactivity is shown for 25- μ l fractions, and the absorbance at 260 nm is shown of a 1:10 dilution of each fraction. Small molecules and soluble proteins were found to elute between fractions 1 and 8, free ribosomes at fractions 13–17, and polysomes from fractions 21–31. The radioactivity in the early fractions includes cellular [³H]AdoMet; radioactivity in the polysome fraction appears to result largely from the incorporation of [³H]methionine (a metabolite of [³H]AdoMet) into nascent polypeptides. *B*, aliquots of selected fractions (150 μ l) were analyzed by SDS gel electrophoresis as shown in Fig. 3 for [³H]methyl esters and total radioactivity in a 3 mM slice containing the 49-kDa region (corresponding to slice 3 in Fig. 3) with a background subtracted of the adjacent 3 mM slice (corresponding to slices 4 and 5 in Fig. 3). Samples for methyl ester determination were counted for a total of 30 min. The data for fraction 0 represent results from analyzing 25 μ l of the material loaded on the gradient.

subjected to strong acid hydrolysis to identify the major non-esterified methylated amino acid species as described under “Experimental Procedures.” The resulting acid hydrolysate was mixed with nonisotopically labeled standards of *N*- ϵ -trimethyl-lysine and 3-methyl-histidine. The mixture was applied to a sulfonated polystyrene-based high resolution cation exchange column (Beckman amino acid analysis resin AA-15, Ref. 20) that separates methyl derivatives of lysine from methyl derivatives of histidine. We found that a major peak of radioactivity co-eluted with the methyl lysine standard, whereas no radioactivity coeluted with the methyl histidine standard (data not shown). Thin layer chromatography of the acid hydrolysis products revealed that 29% of the radioactivity comigrated with the *N*- ϵ -monomethyl-lysine standard, 62% comigrated with the

N- ϵ -dimethyl-lysine standard, and 9% comigrated with the *N*- ϵ -trimethyl-lysine standard. It thus appears that the side chain amino group of lysine residues is a major site of methylation in the 49-kDa polypeptide.

Purification of the Methylated 49-kDa Polypeptide from Cellular Extracts Using Carboxymethyl Cellulose Cation Exchange Chromatography—An *in vivo* [³H]AdoMet-labeled cytosolic extract was fractionated on a CM52 carboxymethyl cellulose column to separate the [³H]methylated 49-kDa polypeptide from other polypeptides (Fig. 2). We found that the [³H]methyl esterified polypeptide eluted both in the flow-through and salt-eluted fractions (Fig. 2). The ratio of base-labile and base-stable material was similar in each of the fractions, indicating that these species comigrated under these conditions. Interestingly,

FIG. 5. Sensitivity of eEF1A methyl ester to hydrolysis catalyzed by proteolytic enzymes. *S. cerevisiae* strain MY101 (30 A_{600 nm} units) was isotopically labeled with [³H]AdoMet, and one-sixth of the resulting lysate was fractionated in each of five parallel lanes by 12% acrylamide SDS gel electrophoresis as described under "Experimental Procedures." Polypeptide bands in the 49-kDa region of dried gel lanes were sliced as illustrated in Fig. 3 and subjected to mild base treatment or protease digestion in reactions buffered with 0.2 M Tris, 0.1 M citrate, pH 6, and 1% Triton X-100 for 24 h at 37 °C as described under "Experimental Procedures." A, portion of the gel containing the 49-kDa polypeptide analyzed for released [³H]methanol using buffer alone (squares), 1 µg/ml trypsin (triangles), 50 µg/ml CPY (circles), or 1 µg/ml chymotrypsin (plus symbols). B, analysis of A extended to include the 42-kDa region (note change in scale of y axis). C, analysis of gel slices for [³H]methyl esters with 1.5 M Na₂CO₃ (diamonds) and for total radioactivity with 30% H₂O₂ (solid line) with a buffer control (squares).



we found that a major 49-kDa Coomassie Blue staining band followed the [³H]methyl radioactivity throughout the column, suggesting that the methylated species might be an abundant polypeptide in the cell. In fact, in the 200 mM NaCl eluted material, the only major Coomassie-staining polypeptide was a 49-kDa species that co-electrophoresed with both the total and methyl ester radioactivity (Fig. 2, fractions 21 and 22). The additional peak of radioactivity (Fig. 2, fraction 30) was not investigated; we detected no Coomassie or silver staining polypeptide. It thus appears that a single abundant 49-kDa polypeptide may contain two types of methyl linkages.

Identification of the Major 49-kDa Coomassie Staining Band as Elongation Factor eEF1A—The purified 49-kDa polypeptide eluted from the CM52 column at 200 mM NaCl (Fig. 2, fraction 21) was given to Dr. Audree Fowler at the UCLA Protein Microsequencing Facility for sequence analysis. Tryptic fragments were separated by high pressure liquid chromatography, and three clear N-terminal sequences were obtained by Edman degradation: NVSVKEI from a peptide eluting at 54 min, IGGIGTV from a peptide eluting at 63 min, and SVEMH-HEQLXQGVPGDNXXFN from a peptide eluting at 68 min (data not shown). Each of these sequences from the 49-kDa polypeptide are identical to those found within *S. cerevisiae* elongation factor 1A (15).

eEF1A is an essential, highly conserved 49–50-kDa polypeptide. It is quite abundant, comprising up to 3% of soluble

protein, and is associated with ribosomes (12, 21). When bound to GTP, eEF1A directs the codon-dependent placement of aminoacyl-tRNA into the A site of the ribosome (12, 22–24). *S. cerevisiae* eEF1A is known to contain *N*-methylated lysine residues; two *N*-ε-monomethyl-lysine residues (positions 30 and 390), a *N*-ε-dimethyl-lysine residue (position 316), and a *N*-ε-trimethyl-lysine residue (position 79) have been characterized (12, 16). The identification of our major methylated 49-kDa polypeptide as elongation factor eEF1A is supported by their common polypeptide size, abundance, and side chain lysine post-translational modifications.

Because eEF1A has a vital role in translation, we decided to see whether the methyl esterified form is associated with ribosomes. We labeled intact yeast cells with [³H]AdoMet and purified ribosomes using sucrose density centrifugation. When these proteins were analyzed by SDS gel electrophoresis, a major 49-kDa Coomassie staining polypeptide was detected corresponding to eEF1A (Fig. 3A). We analyzed gel slices in the region of the 49-kDa polypeptide for [³H]methyl esters and for total radioactivity (Fig. 3B). We found that the major 49-kDa polypeptide in slice 3 also contained the bulk of the total radioactivity in this region (corresponding to the internal lysine *N*-methylation) as well as the bulk of radioactivity as methyl esters (Fig. 3B). These results show that the methyl esterified form of eEF1A does associate with ribosomes.

We then asked whether methyl esterified eEF1A was asso-

TABLE I
Hydrolysis of lysyl methyl ester derivatives catalyzed by proteolytic enzymes

Methyl esterified lysine derivatives were incubated with various proteases, and the products were analyzed by thin layer chromatography. In a final volume of 10 μ l, aliquots (100 nmol) of lysine O-methyl ester (Sigma), N- α -acetyl-lysine O-methyl ester (Sigma), and N- α -benzoyl-lysine O-methyl ester (Bachem Bioscience Inc.) were digested under the same conditions used for the in-gel experiment shown in Fig. 5. After 24 h at 37 °C, 50 nmol of each reaction mixture (5 μ l) was applied to a 20-cm sheet coated with a 0.2-mm layer of silica 60 (EM Separations, Gibbstown, NJ; number 5748). Derivatives were separated with a mobile phase consisting of CH₃OH:~14.8 N NH₄OH (3:1 v/v). The plate was dried overnight at room temperature and then sprayed with ninhydrin (10 mg/ml in acetone) to visualize the positions of the methyl ester and its hydrolysis products. Standards of lysine and N- α -acetyl-lysine were obtained from Sigma; N- α -benzoyl-lysine was obtained from Bachem Bioscience Inc. To determine the fraction hydrolyzed, ninhydrin spots corresponding to the methyl ester and carboxylic acid were quantitated by densitometry using an Alpha Imager 2200 (version 5.04, Alpha Innotech Corp.), and the result was corrected for the relative color yield of standard methyl ester and carboxylic acids. The percentage of hydrolysis shown below was determined by dividing the amount of free carboxylic acid by the sum of free carboxylic acid and methyl ester and multiplying by 100. Reactions were performed in triplicate; the average percentage of hydrolysis \pm the standard deviation is shown.

Incubation condition	Percentage hydrolyzed		
	Lysine O-methyl ester	N- α -Acetyl-lysine O-methyl ester	N- α -Benzoyl-lysine O-methyl ester
Buffer (0.2 M Tris/0.1 M citrate, pH 6, and 1% Triton X-100)	41.8 \pm 3.0	13.3 \pm 4.6	39.1 \pm 1.5
1.5 M Na ₂ CO ₃ , pH 12	97.7 \pm 1.4	97.9 \pm 1.2	96.5 \pm 1.5
Trypsin (1 μ g/ml) in buffer	88.4 \pm 1.2	90.4 \pm 0.3	87.2 \pm 0.3
Carboxypeptidase Y (50 μ g/ml) in buffer	26.0 \pm 1.2	14.1 \pm 4.3	31.6 \pm 1.2
Chymotrypsin (1 μ g/ml) in buffer	30.1 \pm 2.1	12.8 \pm 24.8	19.0 \pm 4.9

ciated with polysomes. We fractionated an extract of cycloheximide-treated *in vivo*-labeled yeast cells by sucrose gradient centrifugation (Fig. 4A). We found little or no methyl ester or total radioactivity in 49-kDa polypeptides in fraction 5, representing the soluble proteins, whereas the methyl esterified 49-kDa polypeptide of eEF1A was found both in the fractions containing free ribosomes (represented by fraction 14) and polysomes (represented by fractions 22–31) (Fig. 4B).

In all of our experiments, we have found that the amount of radioactivity in methyl esters is only 1–5% of that in the stable N-methyl groups on the lysine side chains. It is difficult to calculate a stoichiometry of methyl esterification from these results because the number of stable methyl groups has not been established and because the methyl esterification can occur on proteins that are not labeled on their side chain lysine residues. However, these results suggest that only a small subpopulation of eEF1A proteins are methyl esterified at any one point in time.

Localization of the Carboxyl Methyl Ester on eEF1A in Yeast—To identify whether the carboxyl methyl ester is on an amino acid side chain or at the C terminus of eEF1A, we subjected the [³H]AdoMet-labeled 49-kDa polypeptide to enzymatic digestion using various proteases specific for hydrolysis of α -carboxyl amide (and ester) linkages. These enzymes would not be expected to release [³H]methanol if methylation occurred on a side chain residue in a β - or γ -carboxyl linkage such as those found in aspartyl or glutamyl residues. However, if methylation occurred on the α -carboxyl group at the C terminus, one or more of these proteases might catalyze ester hydrolysis (26–

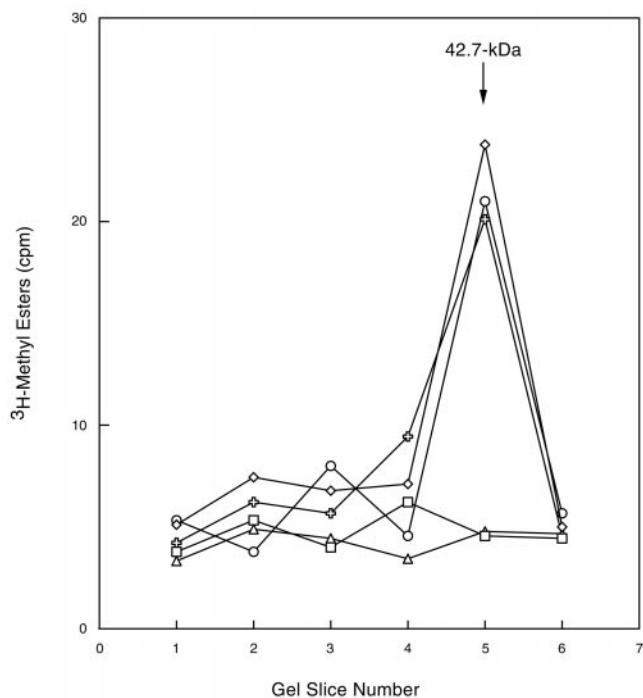


FIG. 6. eEF1A is not methyl esterified *in vitro*. Soluble proteins from *S. cerevisiae* strain JDC9100-2 were isolated as described by Gary *et al.* (20). Protein aliquots (30 μ l) were isotopically labeled *in vitro* with 5 μ l of [³H]AdoMet for 30 min at 30 °C. The reaction was stopped by adding 1 volume of gel electrophoresis sample buffer. Polypeptides were separated using 12% acrylamide SDS gel electrophoresis. [³H]Methyl ester radioactivity was analyzed as described in the legend of Fig. 3.

29). Significantly, we found that [³H]methanol was released when the 49-kDa polypeptide was digested by trypsin but that little or no [³H]methanol was released with CPY, chymotrypsin, or buffer alone (Fig. 5A). The C-terminal amino acid residue of yeast eEF1A is lysine (15). Therefore, trypsin would be expected to cleave C-terminally to this residue and, if it is methyl esterified, release methanol (28, 29). Chymotrypsin, with its specificity for aromatic residues, would not be expected to cleave this linkage.

To confirm the ability of trypsin to catalyze the hydrolysis of a C-terminal lysine methyl ester and the inability of chymotrypsin and CPY to catalyze such hydrolysis, we examined the hydrolysis of O-methyl ester derivatives of lysine, N- α -acetyl-lysine, and N- α -benzoyl-lysine catalyzed by these enzymes using the same conditions as those employed in the gel slice experiment shown in Fig. 5. As shown in Table I, neither CPY nor chymotrypsin digestion resulted in hydrolysis over that seen for samples incubated in buffer alone. On the other hand, trypsin treatment resulted in almost complete hydrolysis, only slightly less than that seen for samples incubated at pH 12. Thus, the data from the *in vivo* experiments shown in Fig. 5 are consistent with the data from the control experiments from Table I and indicate that the methyl ester on the eEF1A C-terminal lysine residue can be hydrolyzed by trypsin but not by CPY and chymotrypsin. The inability of CPY to cleave the lysine α -carboxyl methyl ester under these conditions may reflect the effect of the positively charged side chain. Taken together, these results indicate that the methyl ester linkage on eEF1A is on the carboxyl group of its C-terminal lysine residue.

Utilizing a 12% acrylamide SDS gel system allowed us to resolve a distinct methyl esterified species of about 42-kDa (Fig. 5, B and C, gel slices 5 and 6). Interestingly, the sensitivity of this methyl ester to proteolytic enzymes was exactly opposite to that seen with the eEF1A methyl ester. Here, [³H]methanol

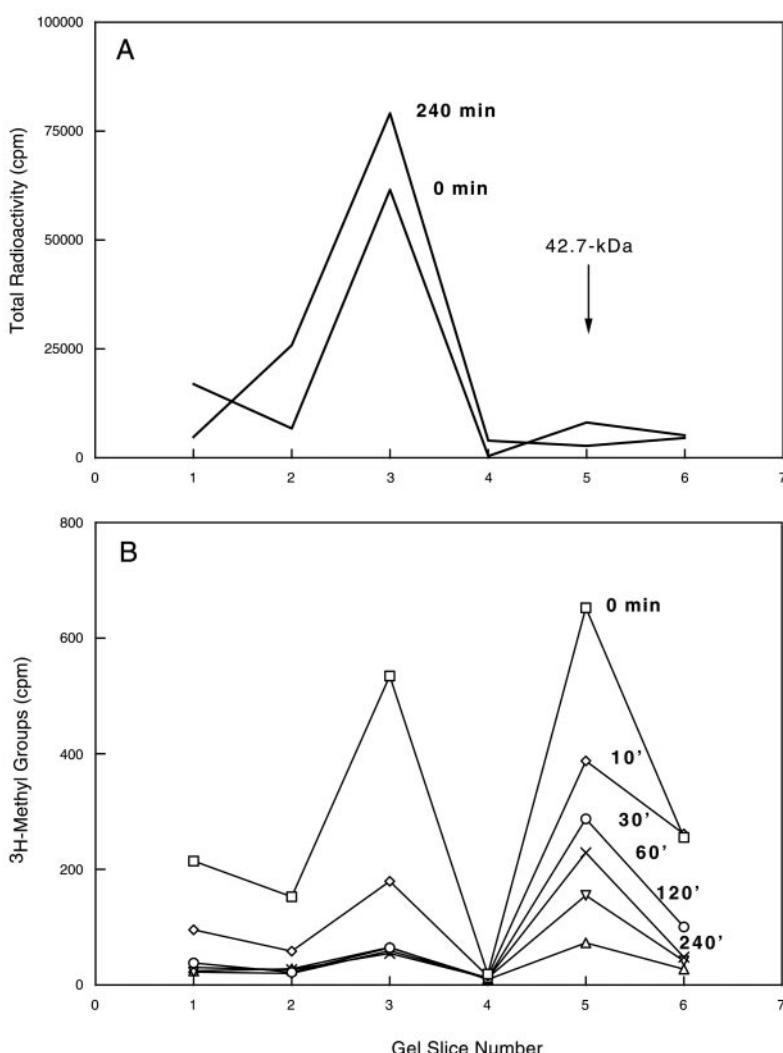


FIG. 7. The eEF1A methyl ester is unstable under physiological conditions. *S. cerevisiae* strain MY101 (30 $A_{600\text{nm}}$ units) was isotopically labeled with [^3H]AdoMet as described under “Experimental Procedures.” The resulting pellet was resuspended in 6 ml of YPD medium containing 1 mM nonisotopically labeled AdoMet. This cell suspension was incubated shaking at 30 °C for up to 4 h. Aliquots (1 ml) were removed at various time points for the preparation of cell lysates. Polypeptides from these lysates were separated by 12% polyacrylamide SDS gel electrophoresis. Gel slices in the range of 40–50 kDa were cut as illustrated in Fig. 3 and analyzed for total radioactivity (A) with 30% H_2O_2 ; [^3H]methyl esters (B) were analyzed with 1.5 M Na_2CO_3 .

is released with CPY or chymotrypsin treatment but is not released with trypsin treatment (Fig. 5B). These results suggest that the 42-kDa polypeptide is methyl esterified on a hydrophobic C-terminal carboxyl group in a reaction clearly distinct from that of eEF1A. Additionally, [^3H]methanol was detected from the 42-kDa polypeptide in an *in vitro* reaction when it was exposed to CPY and chymotrypsin (Fig. 6, slice 5).

An *in vitro* [^3H]methylated 42-kDa polypeptide has been reported previously as the catalytic subunit of yeast protein phosphatase 2A (30, 31). This protein is carboxyl methylated at its C-terminal leucine residue in bovine brain (31), and a similar C-terminal sequence is found in the yeast polypeptide (32). The data presented here are consistent with the identification of the 42-kDa methylated species as the C-terminal methyl esterified form of the catalytic subunit of yeast protein phosphatase 2A.

In all of the experiments presented so far for eEF1A, we examined the products of methylation in intact yeast cells. We now wanted to ask whether the methyl esterification of eEF1A could be detected in an *in vitro* reaction where cell extracts are incubated with [^3H]AdoMet as was demonstrated previously for the 42-kDa polypeptide of protein phosphatase 2A (31). Surprisingly, we found that eEF1A does not appear to be methyl esterified in an *in vitro* reaction mixture (Fig. 6, slice 3), whereas the 42-kDa polypeptide is (Fig. 6, slice 5). This indicates that the C-terminal carboxyl methyltransferase for eEF1A is unstable or inactive under *in vitro* conditions or that eEF1A is not present as an active methyl acceptor.

Methylesterification of eEF1A Is Rapidly Reversible in Intact Yeast Cells—In a pulse-chase experiment, MY101 cells were first labeled *in vivo* with [^3H]AdoMet for 30 min. After the radiolabel was washed away, the cells were then resuspended in medium supplemented with nonisotopically labeled AdoMet and allowed to incubate for up to an additional 4 h. Aliquots of cells were removed, lysed, and analyzed for the presence of [^3H]methyl esters and total radioactivity in the 40–50 kDa region using the gel slice assay. Fig. 7 shows that although the amount of total radioactivity in eEF1A remains relatively constant over a 4-h period (Fig. 7A), the methyl ester linkage is largely lost (Fig. 7B). When intermediate times were analyzed, we found that about 65% of the eEF1A methyl ester is rapidly lost within 10 min after the [^3H]AdoMet was washed out of the medium (Fig. 7B). These results suggest that the level of modification of eEF1A may be modulated in yeast cell physiology.

Finally, our ability to detect the methyl esterification of the 42-kDa species, tentatively identified as the catalytic subunit of protein phosphatase 2A in Figs. 6 and 7, also allows us to monitor the turnover of these methyl groups. We show here that the half-life of the methyl ester on the 42-kDa polypeptide in intact yeast cells is about 20 min, suggesting that it is also reversibly modified and that its extent of methylation may modulate its function as well (Fig. 7).

DISCUSSION

We set out to identify novel methylesterified proteins in yeast and identified a major methylated polypeptide as eEF1A

that is not only amino-methylated on some internal lysine residues but is also carboxyl methylated at the C terminus. The short half-life of the esterified residue in intact cells indicates that this modification is regulated, suggesting that a unique methyltransferase and methylesterase exist and act on this site of the protein.

Elongation factor eEF1A is an essential component of protein synthesis, and its amino acid sequence has been highly conserved throughout evolution (12, 22, 24, 33). Post-translational N-modification of lysine residues has also been found to occur in eEF1A of all organisms studied to date. Methylated *N*-ε-lysine residues have been identified in the prokaryotic counterpart EF1A (formerly EF-Tu) of *Escherichia coli* (34) and *Salmonella typhimurium* (35), as well as in the protist *Euglena gracilis* (36). Eukaryotic organisms including the fungus *Mucor racemosus* (37, 38), brine shrimp *Artemia salina* (39), yeast *S. cerevisiae* (16), and rabbit (40) all contain eEF1A proteins that have been shown to be methylated on *N*-ε-lysine residues as well.

To date, the presence of the methyl esterified form of eEF1A has not been reported. Using models with *A. salina*, investigators have suggested that the C-terminal portion of eEF1A is thought to physically interact with the C termini of GTP-recycling elongation factors (eEF1β or eEF1γ) to promote the efficiency of protein synthesis in the eukaryotic cell (41, 42). Yeast strains containing point mutations in the putative GTP-binding domain of eEF1A exhibit a decreased rate of GTP hydrolysis (43) and subsequently an increased rate of translational infidelity *in vivo* (44). Perhaps the role of eEF1A as a GTPase is regulated by methylation/demethylation of the C-terminal lysine residue.

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