Protein Carboxyl Methylation in *Saccharomyces cerevisiae*: Evidence for STE14-Dependent and STE14-Independent Pathways†

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ABSTRACT: We incubated yeast cells (*Saccharomyces cerevisiae*) with the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine and then fractionated their cellular components by gel electrophoresis in sodium dodecyl sulfate. By analyzing gel slices for [^{3}H]methyl esters by a vapor-phase diffusion assay, we detect major methyl-esterified species that migrate at apparent polypeptide sizes of 24 and 22 kDa and minor species of 49, 38, 35, 33, 31, and 26 kDa. Incubation of extracts from labeled cells with ribonuclease A or proteinase K revealed that the 24- and 22-kDa species represent methyl-esterified RNAs, whereas the other species are methyl-esterified polypeptides. The 38-, 33-, 31-, and 26-kDa polypeptides were not methyl-esterified in an isogenic yeast strain lacking the *STE14* gene encoding a C-terminal isoprenylcysteine methyltransferase, suggesting that they are substrates for the STE14 methyltransferase. On the other hand, the amount of the methylated 49-kDa polypeptide is reduced in the *ste14* mutant, indicating that at least two methylated polypeptides are present—one a substrate of the STE14 methyltransferase and one a substrate of a STE14-independent methyltransferase. The 35-kDa polypeptide also appears to be methylated by a STE14-independent methyltransferase. When cells were incubated in the presence of the protein synthesis inhibitor cycloheximide, little or no methylation of the STE14-dependent species was detected while the methylation of the STE14-independent substrates was unaffected. Pulse–chase studies revealed significant turnover of all of the methylated species in a 4-h period, with the exception of the 38-kDa polypeptide. These results demonstrate the wide range of yeast substrates for the STE14 isoprenylcysteine methyltransferase and suggest the presence of at least one novel protein carboxyl methyltransferase in these cells.

Protein carboxyl methylation and demethylation reactions are of interest because they represent potential mechanisms for achieving cellular regulation by reversible posttranslational modification (Barten & O'Dea, 1990; Hrycyna & Clarke, 1993). For example, chemotactic bacterial cells can regulate the output of their membrane chemoreceptors by the reversible methyl esterification of specific glutamate residues (Simms et al., 1987; Dunten & Koshland, 1991; Borkovich et al., 1992). Of the four known types of enzymes that catalyze protein carboxyl methylation reactions, two have been shown to occur in the yeast *Saccharomyces cerevisiae*. These enzymes include the membrane-bound isoprenylcysteinyl C-terminal methyltransferase encoded by the *STE14* gene that catalyzes the methyl esterification of the **a**-mating pheromone and the RAS1 and RAS2 polypeptides (Hrycyna & Clarke, 1990; Hrycyna et al., 1991; Sapperstein et al., 1994) as well as a newly discovered cytosolic leucyl C-terminal methyltransferase (Xie & Clarke, 1993) that specifically catalyzes the methyl esterification of the 36-kDa catalytic subunit of protein phosphatase 2A in bovine brain (Lee & Stock, 1993; Xie & Clarke, 1994). On the other hand, there is no evidence for the presence of a glutamyl protein methyltransferase catalyzing a reaction similar to that seen in chemotactic bacteria, nor is there evidence for the presence of the L-isoaspartyl/D-aspartyl methyltransferase found in most eucaryotic and procaryotic cells that can initiate the repair of proteins containing altered L-isoaspartyl and D-aspartyl residues (Li & Clarke, 1992a,b).

We have been interested in characterizing new protein methylation reactions in yeast that may include L-glutamate methyl ester formation or novel types of chemistry. The understanding of such reactions may provide further insights into cellular strategies for regulating their metabolic pathways and for controlling processes such as signal transduction and cell division. The advantages of well-characterized genetic approaches in the yeast *S. cerevisiae* have made these cells an attractive model for such studies of protein methylation. For example, analysis of mutants of the STE14 gene revealed defects in the processing of the RAS1 and RAS2 precursors (Hrycyna et al., 1991) as well as **a**-specific sterility (Marcus et al., 1991; Sapperstein et al., 1994). These defects are due to the failure of the cells to methyl esterify the C-terminal farnesylcysteine residue of the RAS polypeptides or the **a**-mating pheromone peptide, respectively. As a first step in our present work, we have analyzed the full complement of the major methyl-esterified polypeptides in intact yeast cells. We report here evidence for additional substrates for the STE14 C-terminal isoprenylcysteinyl methyltransferase, as well as data suggesting that one or more potentially novel protein carboxyl methyltransferases function in these cells.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions. *S. cerevisiae* strains SM1058 (*MAT***a** *trp1 leu2 ura3 his4 can1 STE14*$^+$) and SM1188 (*ste14*Δ*::TRP1*, otherwise isogenic to SM1058)

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were obtained from Dr. Susan Michaelis, Johns Hopkins University, and are described in Hrycyna et al. (1991). Strain ABYS1 (*MATa pra1 prb1 prc1 cps1 ade STE14*$^+$) is described in Achstetter et al. (1984). Cells were grown at either 30 or 37 °C in YPD medium [1% (w/v) yeast extract (Difco Laboratories), 2% (w/v) Bacto-Peptone (Difco), and 2% (w/v) D-glucose] to an OD_{600nm} between 0.6 and 0.8.

Isotopic Labeling. Cells in exponential growth phase (5 OD_{600nm} units) were spun at 1600*g* for 6 min in 15-mL polystyrene centrifuge tubes at room temperature. Cells were resuspended in 0.82 mL of YPD medium and mixed with 0.18 mL of [^{3}H]AdoMet[1] [NEN NET-155H, 100 μCi, 73 Ci/mmol, in 9:1 dilute H_2SO_4 (pH 2.0)/ethanol] to give a final concentration of [^{3}H]AdoMet of 1.4 μM. Cells were incubated for various times at 30 or 37 °C with shaking and were then transferred to 1.5-mL polypropylene microcentrifuge tubes and spun at 13600*g* for 1 min, washed with 1 mL of H_2O, and spun again at 13600*g* for 1 min. In experiments where cycloheximide was used to inhibit protein synthesis, a stock of 10 mg/mL reagent in ethanol was prepared and added to the incubation mixture to give a final concentration of 100 μg/mL.

Dodecyl Sulfate Gel Electrophoresis and Analysis of Methyl-Esterified Polypeptides. The cell pellets from the isotopic labeling incubations were each resuspended in 50 μL of 1% sodium dodecyl sulfate/0.67 mM PMSF. PMSF was prepared as a 100 mM stock in ethanol. Baked zirconium beads (0.2 g) were added, and the mixture was heated at 100 °C for 2 min. Each sample was then vortexed in 1-min bursts for a total of 7 min at 4 °C, and the sample was then heated at 100 °C for an additional 3 min. Approximately 40 μL of this extract was removed with a plastic micropipet tip and mixed with 40 μL of a concentrated gel electrophoresis sample buffer [35.5% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, 6.0% (w/v) sodium dodecyl sulfate, 0.18 M Tris-HCl, pH 6.8, 0.005% (w/v) bromophenol blue]. Alternatively, in control experiments, the extract was treated at this point with proteinase K or ribonuclease. Extracts containing approximately 100 μg of protein were incubated with either 40 μg of bovine pancreatic ribonuclease (Sigma, heated at 100 °C for 15 min) or 20 μg of proteinase K (Sigma) at room temperature for 30 min. Samples (12 μL, containing approximately 100 μg of protein) were applied to alternate wells (0.75 cm wide by 1.5 cm high) in a 4 cm long stacking gel [4.5% (w/v) acrylamide, 0.15% *N*,*N*-methylenebis(acrylamide)] prepared in the Laemmli buffer system (Laemmli, 1970). Polypeptide molecular mass standards (2 μL) [Bio-Rad 161-0304, low molecular weight, containing 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) each at a concentration of about 2 mg/mL] were mixed with 40 μL of the sample buffer described above, and 10 μL of the mixture was applied to wells between each of yeast samples. After electrophoresis of the tracker dye through the stacking gel at 70 V, the voltage was increased to 160 V for electrophoresis though the separating gel [1.5 mm thick by 12 cm in length; 10% (w/v) acrylamide, 0.34% *N*,*N*-methylenebis(acrylamide)]. The gel was stained with 0.1% Coomassie Brilliant Blue (w/v) in 50% methanol (v/v)/10% acetic acid (v/v) for 90 min and then destained overnight in 10% acetic acid (v/v)/5% methanol (v/v). After 30 min in 5% glycerol (v/v), the gel was dried onto filter paper backing at 60 °C.

Dried gel lanes were cut into 39–42 slices 0.3 cm long by about 1 cm wide. To release [^{3}H]methyl groups from polypeptide methyl esters, slices were mixed with 250 μL of 1 M sodium carbonate, pH 11.3, in 1.5-mL polypropylene microcentrifuge tubes. Control experiments indicated that the final pH of these samples varied between pH 10.5 and 10.8, presumably due to a small residual amount of acetic acid in the dried gel slice. These tubes were gently floated in 20-mL plastic scintillation vials containing 5 mL of Safety Solve counting fluid (Research Products International, Mt. Prospect, IL). After incubation for 24 h at 37 °C in sealed vials to allow [^{3}H]methanol to diffuse through the vapor phase and accumulate in the counting fluid, each vial was counted.

Protein Analysis. Samples (up to 0.1 mL) were mixed with 1.0 mL of 10% (w/v) of trichloroacetic acid, incubated at room temperature for 10 min, and then spun for 10 min at 13600*g* in microcentrifuge tubes. After the supernatant was decanted, the protein pellet was analyzed by a modified Lowry procedure (Bailey, 1967) using a standard of bovine serum albumin.

Cell Fractionation. This procedure was modified from that of Powers et al. (1986). Labeled cells were washed once in water and resuspended in 50 μL of 50 mM potassium phosphate, 150 mM sodium chloride, 1 mM β-mercaptoethanol, and 1 mM PMSF, pH 7.4, and disrupted by vortexing for 7 min in 1-min pulses with 0.25 g of baked zirconium beads. After the extract was removed with 0.5 mL of this buffer, cell debris was removed by centrifugation at 1000*g* for 10 min at 4 °C. The remaining supernatant was then either directly prepared for SDS gel electrophoresis ("total extract") or separated into soluble and membrane fractions. In the former case, 200 μL of the supernatant was diluted with 10 μL of 20% (w/v) Triton X-100 and 200 μL of 50 mM potassium phosphate, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 1 mM β-mercaptoethanol, and 1 mM PMSF, pH 7.4, and 39 μL was mixed with 21 μL of SDS sample buffer as described above. In the latter case, 300 μL of the supernatant was diluted with 300 μL of 50 mM potassium phosphate, 150 mM sodium chloride, 1 mM β-mercaptoethanol, and 1 mM PMSF, pH 7.4, and centrifuged at 153000*g* for 60 min at 4 °C in a Beckman Type 65 rotor. The supernatant from this step was used as the "soluble" fraction; 51 μL was mixed with 26 μL of SDS sample buffer. The pellet from this step was resuspended in 100 μL of SDS sample buffer and 150 μL of 50 mM potassium phosphate, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 1 mM β-mercaptoethanol, and 1 mM PMSF, pH 7.4, and heated at 100 °C for 5 min; 62 μL of this preparation was used for gel electrophoresis.

RESULTS

Detection of Protein Methyl Esters in Polypeptides of Yeast Cells. Taking advantage of the permeability of *S. cerevisiae* cells to the biological methyl donor AdoMet via a high-affinity transport system (Murphy & Spence, 1972), we incubated yeast cells with [^{3}H]AdoMet and then analyzed extracts by polyacrylamide gel electrophoresis in SDS. Here, we prepared the separating gel in the pH 8.8 buffer system described by Laemmli (1970) to minimize the contribution of any L-isoaspartyl α-methyl esters or D-aspartyl β-methyl esters that may have been formed by an L-isoaspartate–(D-aspartate)

[1] Abbreviations: [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; AdoMet, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

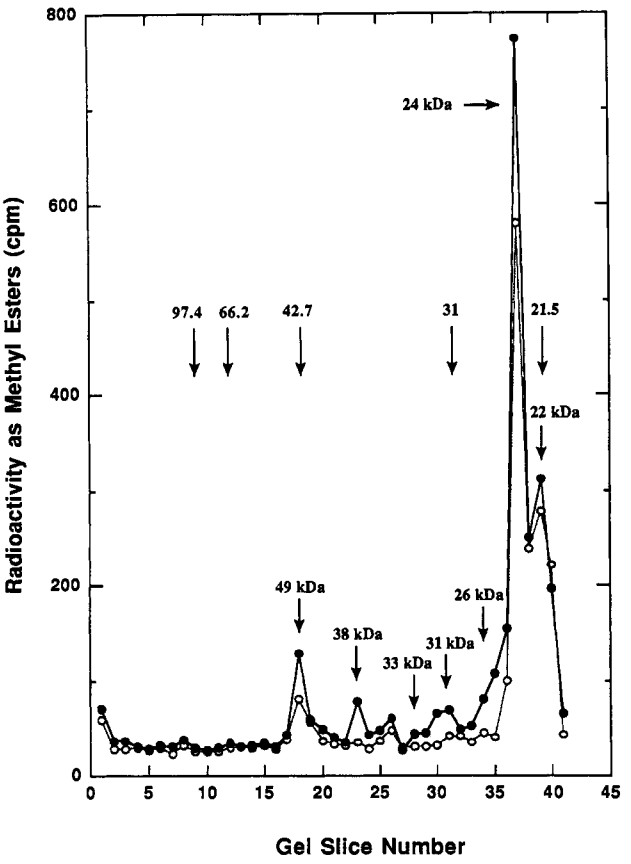

FIGURE 1: Methyl esterification of yeast macromolecules in intact cells labeled with [^{3}H]AdoMet. *S. cerevisiae* strains SM1058 (*STE14*$^+$; filled symbols) and SM1188 (*ste14*$^-$; open symbols) were grown at 37 °C and incubated with 1.4 μM [^{3}H]AdoMet for 15 min at 37 °C as described in Experimental Procedures. Polypeptides were separated by SDS gel electrophoresis and gel slices analyzed for [^{3}H]methyl esters using base hydrolysis in pH 11.3 sodium carbonate buffer as described in Experimental Procedures. The positions of the molecular mass markers electrophoresed in parallel lanes are indicated in the center of the figure, and the calculated polypeptide molecular masses of the [^{3}H]methylated products are shown with the corresponding arrows. The small peak of radioactivity seen in both strains here between the 38- and 33-kDa methylated polypeptides migrates at a position of about 35 kDa. In other experiments where the region between the 21.5- and 14.4-kDa molecular mass standards was examined, no significant peaks of [^{3}H]methyl-esterified polypeptides were detected.

methyltransferase (Terwilliger & Clarke, 1981), while preserving the more stable L-glutamyl (Kleene et al., 1977), C-terminal isoprenylcysteinyl (Stephenson & Clarke, 1990) and C-terminal leucyl (Xie & Clarke, 1993) methyl esters. We analyzed [^{3}H]methyl esters by a vapor-phase diffusion assay where slices of dried gels are treated with base to release [^{3}H]methanol. Because strong base treatment of polypeptides [^{3}H]methylated at the guanidino group of arginine residues can release small amounts of volatile [^{3}H]methylamine (Paik & Kim, 1980), we performed the hydrolysis step with 1 M sodium carbonate at pH 11.3, conditions where no contribution from [^{3}H]methylated arginine residues would be expected [cf. Najbauer et al. (1991)].

This type of analysis revealed that yeast cells contain a number of methyl-esterified species (Figure 1). We detected two major species of 24 and 22 kDa and minor species of 49, 38, 35, 33, 31, and 26 kDa. To ensure that our vapor-phase assay conditions resulted in the complete hydrolysis of methyl esters, we performed control experiments where gel slices were incubated in the pH 11.3 sodium carbonate buffer for 9 days rather than 1 day at 37 °C. Under these conditions, the pattern of methylated species was identical. We found no significant changes in the radioactivity incorporated into the 38-, 35-, 33-, 31-, and 24-kDa polypeptides and at most a 2-fold increase in the radioactivity incorporated into the 49-, 26-, and 22-kDa species (data not shown). We also performed control experiments where methyl ester hydrolysis of components in gel slices was carried out in 6 M HCl rather than the sodium carbonate buffer (Najbauer et al., 1991) and obtained similar results (data not shown). Finally, we analyzed gel slices under conditions where methyl esters were hydrolyzed under highly basic conditions where 1 M sodium hydroxide replaced the pH 11.3 sodium carbonate buffer. Under these conditions, a very different pattern of methylated species was observed with major methylated components at 56 and 35 kDa largely obscuring the methylated species described above (data not shown). These former species presumably reflect polypeptides methylated at arginine residues that generate [^{3}H]methylamine upon strong base hydrolysis (Paik & Kim, 1980; Najbauer et al., 1991).

We then performed experiments to verify that the radiolabeled species observed in gel slices represented protein methyl esters. It has been reported that yeast arginyl $tRNA_{III}$ is modified by reversible methyl esterification of a 5-(carboxymethyl)uridine residue (Bronskill et al., 1972; Kuntzel et al., 1975). Since it is possible that methyl-esterified tRNA species may migrate on SDS gel electrophoresis along with polypeptide components, we analyzed radiolabeled extracts that were treated with either ribonuclease A or with proteinase K. We found that the Coomassie Blue staining patterns of gel lanes loaded with ribonuclease-treated extracts were similar to control staining patterns, with the exception of an additional band at 17 kDa corresponding to the polypeptide size of ribonuclease. On the other hand, treatment of extracts with proteinase K resulted in the loss of all Coomassie Blue staining with the exception of a band at 33 kDa corresponding to the proteinase itself. When gel slices from these lanes were analyzed for [^{3}H]methyl esters, we found that ribonuclease treatment resulted in the quantitative loss of the 24- and the 22-kDa species, but no changes in the other radiolabeled bands (Figure 2). On the other hand, proteinase K treatment resulted in the loss of all radioactive bands with the exception of the 24- and 22-kDa species (Figure 2). These results clearly indicate that the [^{3}H]methyl-esterified 24- and 22-kDa species represent methyl-esterified RNA molecules, whereas the other radiolabeled species represent methyl-esterified proteins.

We then compared the methyl esterification of yeast components when an isogenic strain (SM1188) lacking the *STE14* gene for the C-terminal isoprenylcysteine methyltransferase was incubated with [^{3}H]AdoMet (Figure 1). In this experiment shown and in additional similar experiments, we found that methyl esterification of the RNA species or the 35-kDa polypeptide was largely unaffected, while methylation was significantly reduced in the 49-kDa polypeptide and was not detectable in the 38-, 33-, 31-, and 26-kDa polypeptides. These results indicate that distinct (and potentially novel) protein methyltransferases catalyze the modification of a subset of the 49-kDa polypeptide and the 35-kDa polypeptide, while the STE14 methyltransferase is responsible for the other polypeptide species observed. In the experiment shown in Figure 1, yeast strains were grown and incubated with [^{3}H]-AdoMet at 37 °C; in other experiments, cells grown and incubated at 30 °C gave similar results.

Effect of Protein Synthesis Inhibitors on Methyl Esterification. To determine whether the methyl esterification reactions seen in Figure 1 occur cotranslationally or post-

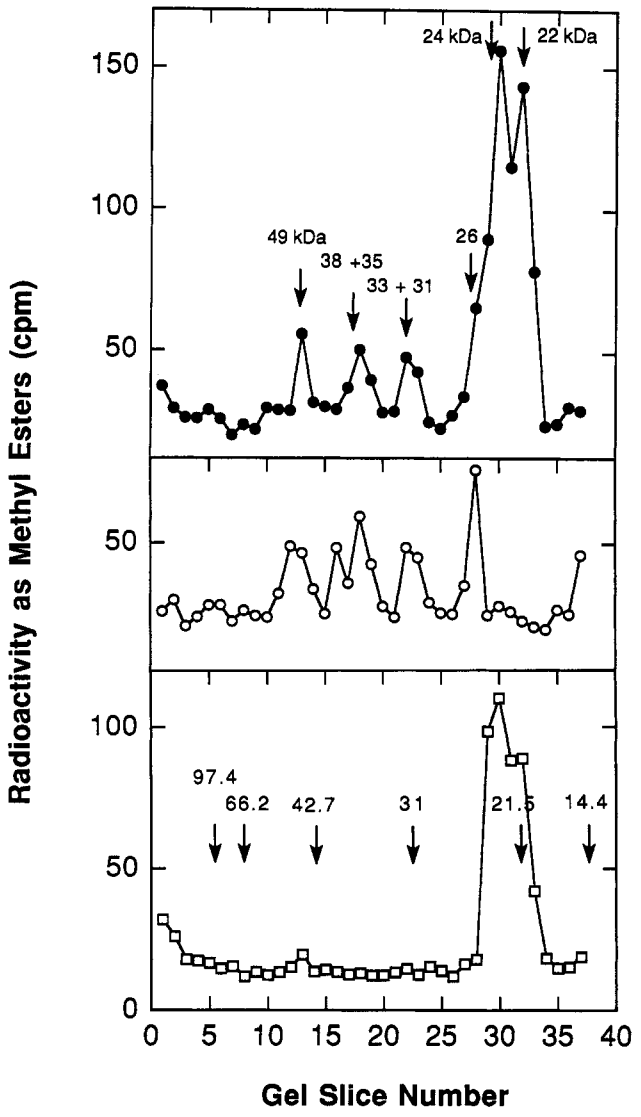

FIGURE 2: Effect of proteinase K and ribonuclease A treatment of [^{3}H]methyl-esterified yeast macromolecules labeled with [^{3}H]-AdoMet in intact cells. *S. cerevisiae* strain SM1058 (*STE14*$^+$) was grown at 30 °C and incubated with 1.4 μM [^{3}H]AdoMet for 30 min at 30 °C as described in Experimental Procedures. Control extracts (filled circles), or extracts treated with ribonuclease (open circles) or proteinase K (open squares) as described in Experimental Procedures, were fractionated by SDS gel electrophoresis and gel slices analyzed for [^{3}H]methyl esters as shown in Figure 1.

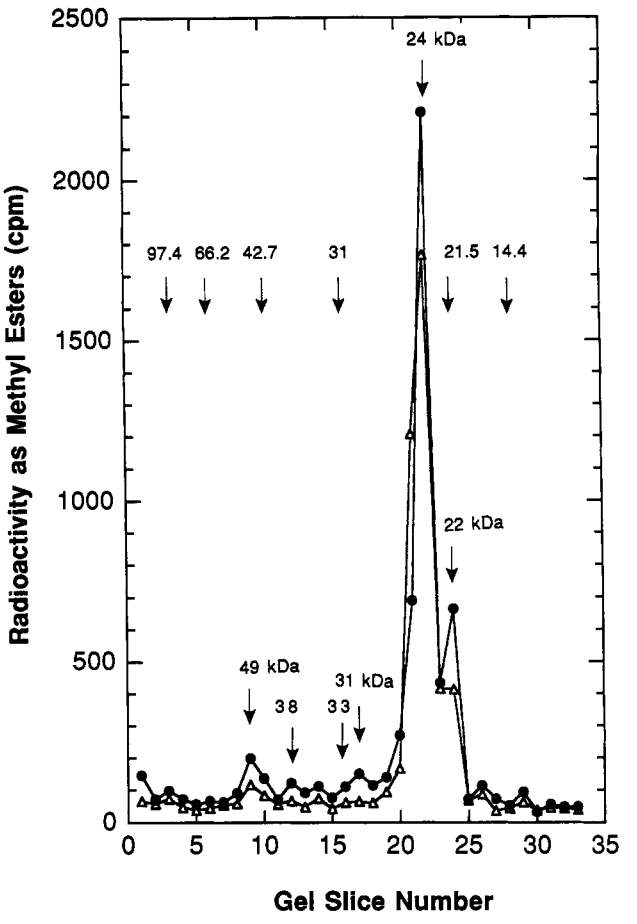

FIGURE 3: Effect of inhibiting protein synthesis on macromolecular methyl esterification in yeast. *S. cerevisiae* strain SM1058 (*STE14*$^+$) was grown at 30 °C and incubated with 1.4 μM [^{3}H]AdoMet in the presence (open triangles) and absence (closed circles) of 100 μg/mL cycloheximide for 5 min as described in Experimental Procedures. Polypeptides were separated by SDS gel electrophoresis and gel slices analyzed for [^{3}H]methyl esters using base hydrolysis in pH 11.3 sodium carbonate buffer as described in Experimental Procedures, except that the resolving gel was made from 11.5% acrylamide/0.4% *N*,*N*-methylenebis(acrylamide). The positions of the molecular mass markers electrophoresed in parallel lanes are indicated in the center of the figure. Methylated species corresponding to those identified in Figure 1 are indicated by arrows. In this experiment we were unable to resolve the 26-kDa component from the 24-kDa peak. We did detect, however, a 35-kDa methylated species corresponding to that seen in Figure 1. Experiments conducted where the incubation time was increased to either 20 or 60 min gave quantitatively and qualitatively similar results (data not shown).

translationally, yeast cells were incubated for various times in the presence and absence of cycloheximide, a protein synthesis inhibitor. As shown in Figure 3, this treatment had only a small effect on the methylation of the RNA species. On the other hand, methylation was significantly reduced in the 49-, 38-, 33-, and 31-kDa species. Interestingly, the pattern of methylation seen in the presence of the protein synthesis inhibitor was very similar to that seen when yeast cells lacking the STE14 methyltransferase were examined. These results suggest that the bulk of STE14-dependent methylation occurs shortly after protein synthesis and proceeds to completion. Because of the possibility that changes in intracellular methionine levels with cycloheximide treatment may alter the specific activity of the [^{3}H]AdoMet label via dilution by endogenous synthesis from this amino acid, we performed control experiments that demonstrated that methionine added to the medium in concentrations up to 0.75 mM did not affect the [^{3}H]methyl esterification of either the RNA or protein species (data not shown).

Turnover of Methylated Polypeptides in Intact Cells. To determine whether the methyl esterification of yeast cell macromolecules is a reversible reaction, we pulse-labeled SM1058 (*STE14*$^+$) cells with [^{3}H]AdoMet for 10 min and then chased the cells in an incubation of 240 min in the presence of a 700-fold excess of non-isotopically-labeled AdoMet. As shown in Figure 4, a range of stabilities was observed. Little or no change in the methylation level of the 38-kDa STE14-dependent substrate was observed over the 4-h chase period, indicating that the methyl esters on these polypeptides were relatively stable. On the other hand, methyl esters were lost from the methylated RNA migrating with an apparent polypeptide size of 24 kDa with an approximate half-time of 3 h, and from the 22-kDa methylated RNA with a much longer half-time. Turnover was also detected in the 49- and the 33/31-kDa methylated polypeptides. Such turnover can reflect both methyl ester hydrolysis and polypeptide degradation.

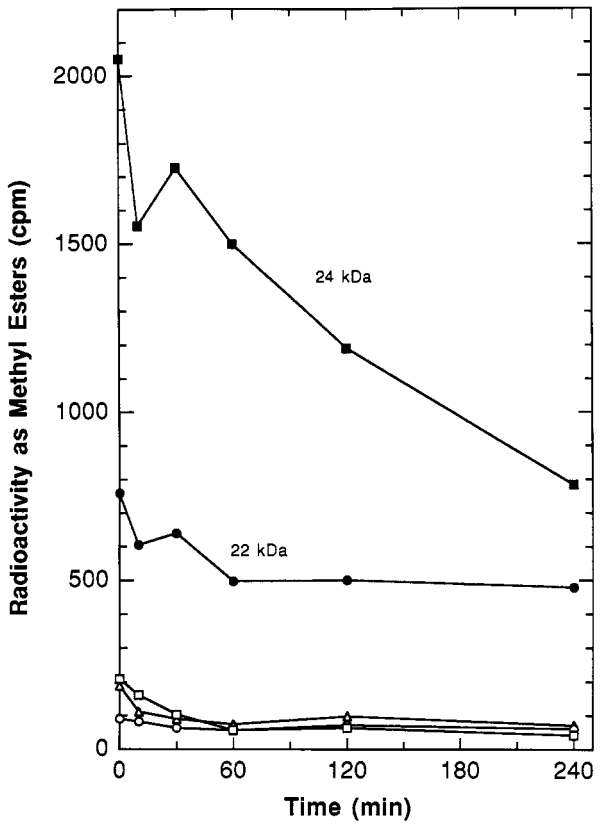

FIGURE 4: Pulse–chase analysis of methyl ester turnover of yeast macromolecules. *S. cerevisiae* strain SM1058 (*STE14*$^+$) was grown in 75 mL of YPD media at 30 °C to an OD_{600nm} of 0.71. Cells (30 OD_{600nm} units) were resuspended in 4.9 mL of YPD medium containing 1 mM L-methionine and mixed with 1.08 mL of [^{3}H]-AdoMet to give a final concentration of [^{3}H]AdoMet of 1.4 μM. After incubation at 30 °C for 10 min, cells were pelleted at 1240*g* for 10 min at 4 °C and then resuspended in 3 mL of YPD media containing 1 mM L-methionine and 1 mM non-isotopically-labeled AdoMet (Sigma, iodide salt). An aliquot of 0.5 mL (zero time point) was immediately removed to 0 °C, and cells were collected by centrifugation as described in Experimental Procedures. The remainder of the material was incubated at 30 °C, and further aliquots were taken after 10, 30, 60, 120, and 240 min. All cells were then processed for SDS gel electrophoresis as described in Experimental Procedures. The total radioactivity as methyl esters in the 49-kDa (open squares), 38-kDa (open circles), 33- and 31-kDa (open triangles), 24-kDa (closed squares), and 22-kDa (closed circles) methylated species is shown as a function of the time of the chase in non-isotopically-labeled AdoMet. In these experiments, it was not possible to separate the radioactivity in the 33- and 31-kDa species, and the 26-kDa species was not sufficiently resolved from the 24-kDa peak to permit analysis.

Localization of Methyl-Esterified Polypeptides in Soluble and Membrane Fractions. When intact yeast cells are incubated with [^{3}H]AdoMet and then separated into soluble and membrane fractions, we found that the 38-, 33/31-, and 26-kDa STE14-dependent methyl acceptors were localized exclusively to the membrane fraction, while the RNA methyl acceptors were largely confined to the soluble fraction (Figure 5). The 49-kDa methyl acceptor was found equally distributed between the soluble and membrane fractions.

In Vitro Methylation Reactions. We finally asked whether we could reproduce the pattern of protein carboxyl methylation by incubating membrane and soluble fractions of *S. cerevisiae* with [^{3}H]AdoMet. To minimize the potential proteolysis and esterolysis that can be catalyzed by vacuolar proteases and peptidases released during cell breakage (Jones, 1991), we utilized in these experiments the ABYS1 strain that lacks vacuolar proteinase A and B and carboxypeptidase Y and S

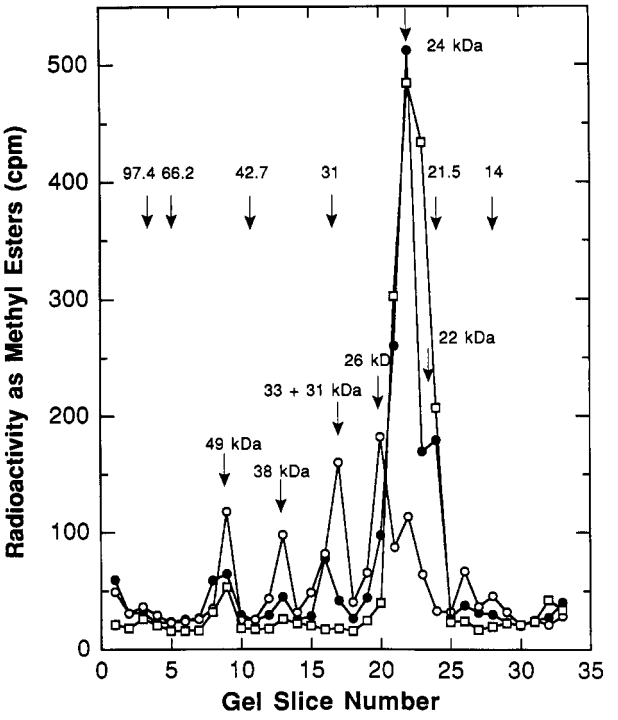

FIGURE 5: Distribution of methyl-esterified yeast macromolecules in soluble and membrane fractions. *S. cerevisiae* strain SM1058 (*STE14*$^+$) was grown at 30 °C, and 6 $OD_{600\ nm}$ units of exponentially-growing cells were incubated with 1.4 μM [^{3}H]AdoMet for 10 min as described in Experimental Procedures. "Total extract", soluble, and membrane fractions were then prepared as described in Experimental Procedures. Polypeptides were separated by SDS gel electrophoresis and gel slices analyzed for [^{3}H]methyl esters using base hydrolysis in pH 11.3 sodium carbonate buffer as described in Experimental Procedures, except that the resolving gel was made from 11.25% acrylamide/0.39% *N,N*-methylenebis(acrylamide). The positions of the molecular weight markers electrophoresed in parallel lanes are indicated in the center of the figure. Methylated species corresponding to those identified in Figure 1 are indicated by arrows. Samples from the "total extract" lane (39 μg of protein) are indicated with closed circles, while samples from the soluble fraction (21 μg of protein) are shown in open squares and samples from the membrane fraction (31 μg of protein) are shown in open circles.

activity (Achstetter et al., 1984). As shown in Figure 6, we detect little protein carboxyl methylation with the membrane fraction. On the other hand, incubation of the cytosolic fraction with AdoMet results in the methyl esterification of substrates migrating at the position of the RNA methyl acceptors as well as a major 43-kDa polypeptide and a 35-kDa polypeptide (Figure 6). The [^{3}H]methyl-esterified 43-kDa methylated polypeptide has not been observed in the experiments where intact cells are labeled (cf. Figures 1, 3, and 5). It may represent either a degradation product of the 49-kDa methyl acceptor or a new species that is not methylated in intact cells under the conditions used in our experiments. Possible candidates for such new species include the catalytic subunits of the two *S. cerevisiae* protein phosphatase 2As with molecular weights of 41 941 and 43 050, respectively, for the *PPH21* and *PPH22* gene products (Peng et al., 1991; Sneddon et al., 1990). The mammalian homologs of these polypeptides are methylated under similar conditions at their C-terminal leucyl residues (Xie & Clarke, 1993, 1994), and a similar reaction has been shown to occur in yeast (Xie & Clarke, 1993). When the cytosolic fraction of yeast strains SM1058 and SM1188 is incubated with [^{3}H]AdoMet, methyl esterification is greatly reduced, consistent with the action of proteases in disrupted cells with a wild-type complement of vacuolar proteases.

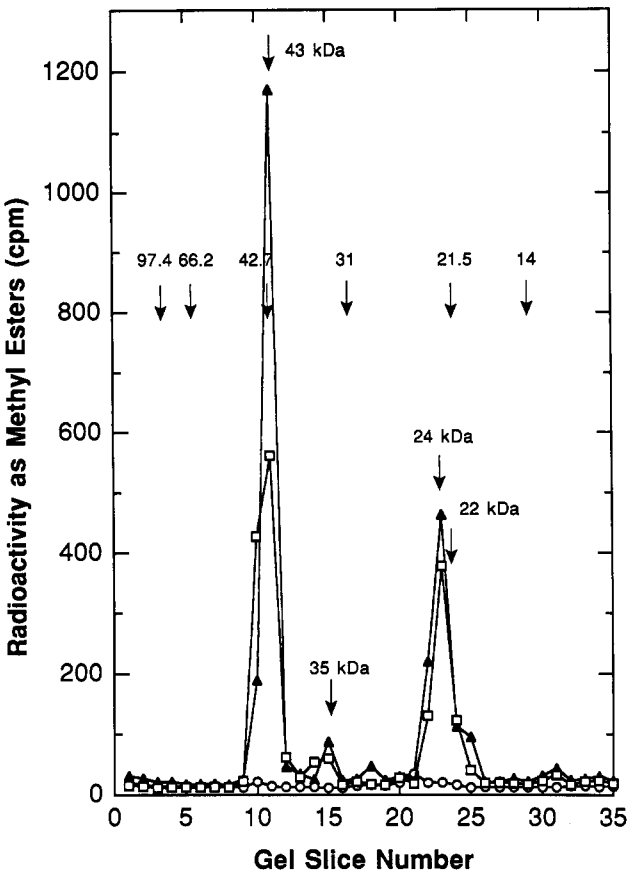

FIGURE 6: In vitro methylation of soluble and membrane fractions of *S. cerevisiae* strain ABYS1. Cell fractions, prepared as described in Experimental Procedures, were incubated in 0.75 μM [^{3}H]AdoMet (73 Ci/mmol) and 50 mM Tris-HCl, pH 7.5, at 30 °C for 30 min. Aliquots were then prepared for SDS gel electrophoresis as described in Experimental Procedures. The positions of the molecular weight markers electrophoresed in parallel lanes are indicated in the center of the figure. Radioactivity as [^{3}H]methyl esters produced in the proteins of the soluble fraction alone (123 μg of protein) is shown in open squares, that produced from the membrane fraction alone (62 μg protein) is shown as open circles, and that produced from a mixture of the soluble (123 μg of protein) and membrane (62 μg of protein) fraction is indicated by closed triangles.

Finally, we performed in vitro methylation experiments where the soluble and membrane fractions were reconstituted and incubated with [^{3}H]AdoMet. Here, the pattern of methyl-esterified polypeptides observed was similar to that seen when the soluble fraction was incubated in the absence of the membrane fraction (Figure 6). Control experiments where the membrane fraction from SM1058 and SM1188 strains were incubated in place of the membrane fraction from ABYS1 cells gave similar results, indicating that the membrane-bound STE14 methyltransferase was not contributing to the methylation pattern observed (data not shown). The absence of methylated products of the STE14 methyltransferase in these in vitro experiments may reflect the stoichiometric methylation of STE14-dependent methyl acceptors in the cell so that free α-carboxyl groups of C-terminal isoprenylcysteine residues are only available for the enzyme shortly after their biosynthesis.

DISCUSSION

Identification of Methyl-Accepting Substrates in Intact Yeast Cells. The experiments described in this report show that *S. cerevisiae* cells contain at least three groups of substrates for macromolecular carboxyl methyltransferases. The first group includes the soluble RNA species that elute at the positions expected for 24- and 22-kDa polypeptides and that are methylated in reactions independent of the STE14 C-terminal isoprenylcysteine methyltransferase. These two RNA species represent the major sites of carboxyl methylation in these cells. The methylation of these species is independent of protein synthesis, and evidence is presented for the turnover of methyl groups on the 24-kDa species with a half-time of approximately 3 h. At least a portion of these RNA molecules include the arginyl $tRNA_{III}$ that has been shown to be reversibly methylated on a covalently modified uridine residue (Bronskill et al., 1972; Kuntzel et al., 1975). The physiological role of this RNA modification reaction is not understood.

Second, we have identified 49- and 35-kDa STE14-independent methyl-accepting polypeptides. It has been difficult to further characterize these species because the former polypeptide comigrates with a 49-kDa substrate of the STE14 methyltransferase (see below), while the latter methylated polypeptide is present in relatively low concentrations. The ability to methylate a 35-kDa species in vitro, however, may aid in the identification of the methyltransferase responsible for its modification (Figure 6).

The final group of methyl-accepting substrates are products of the STE14 C-terminal isoprenylcysteine methyltransferase. These include 49-, 38-, 33-, 31-, and 26-kDa polypeptides. The 38-, 33-, 31-, and 26-kDa substrates are localized in membranes, and their methylation is tightly linked to protein synthesis. While we find no evidence for the significant turnover of the 38-kDa polypeptide, a slow turnover is observed for the 33/31-kDa species. These results suggest, at least under the experimental conditions described here, that the C-terminal isoprenylcysteine methylation reaction occurs shortly after polypeptide synthesis and that the modification of these protein species is only slowly reversible.

The *STE14*-encoded C-terminal isoprenylcysteine methyltransferase has been shown to catalyze the methyl esterification of the **a**-mating pheromone and the RAS1 and RAS2 polypeptides (Hrycyna et al., 1991; Sapperstein et al., 1994). Although the peptidyl **a**-mating pheromone would not be expected to be retained on the gel system employed in these experiments, methyl-esterified RAS polypeptides would be detected if present in sufficient amounts. The precursors for the RAS1 and RAS2 polypeptides have molecular masses of 34 303 and 34 689 daltons. However, the modification of these polypeptides by the cleavage of the three C-terminal residues and the farnesylation and palmitoylation of the two newly-exposed C-terminal cysteine residue result in apparent sizes on SDS gel electrophoresis of about 36 kDa for RAS1 (Fujiyama & Tamanoi, 1986) and 38 kDa (Deschenes et al., 1989), 39 kDa (Hrycyna et al., 1991), and 40 kDa for RAS2 (Fujiyama & Tamanoi, 1986). Although it is difficult to exactly correlate polypeptide molecular masses determined in different laboratories, the RAS2 polypeptide [synthesized at much higher levels than the RAS1 polypeptide (Breviario et al., 1988)] is probably at least one component of the 38-kDa methylated species described in this work.

Other yeast polypeptides with precursor C-terminal -CXXX species that would be expected to be methylated by the STE14 enzyme include the *RHO1*, *RHO2*, *BUD1/RSR1*, *STE18*, and *CDC42* gene products (Hrycyna & Clarke, 1991), as well as the newly described *RHO3* and *RHO4* products (Matsui & Toh-e, 1992). The BUD1/RSR1 polypeptide has a precursor size of 30 390 daltons and may make up all or part of the 31-kDa species detected here. The CDC42, RHO1, and RHO2 polypeptides have precursor molecular masses of 21 353, 23 172, and 21 462 daltons, respectively, and thus

may be obscured under the large peak of the STE14-independent methyl-accepting 24-kDa species. The mature RHO1 protein has been found to migrate as a 23-kDa species on SDS gel electrophoresis and has been localized to the Golgi apparatus (McCaffrey et al., 1991). Interestingly, we do detect membrane-bound methylated polypeptides in this molecular weight range in the fractionation experiment shown in Figure 5. The mammalian homolog of the yeast CDC42 protein, the small G-protein G25K, has been shown to be a cytosolic species that is translocated to the membrane upon methyl esterification (Backlund, 1992). The RHO3 and RHO4 precursor polypeptides have molecular weights of 25 316 and 25 641, respectively, and their methylated forms may elute in the 26-kDa region on the gel. Finally, we find no evidence for a methylated species corresponding to the 12 625-dalton precursor of the *STE18* gene product. Further studies will be required to determine what fraction of the STE14-dependent radioactivity seen in these experiments is due to the methyl esterification of these polypeptides and how much is due to that of presently unidentified substrates for the STE14 methyltransferase.

Evidence for a Novel Type of Protein Carboxyl Methylation Reaction. The work described here demonstrates that the methyl esterification of a portion of the 49-kDa species and the 35-kDa species reflects reactions not characteristic of any of the four known types of protein carboxyl methyltransferases. Two of these enzymes have not been shown to be present in yeast, including an L-glutamate methyltransferase specific for membrane-bound bacterial chemoreceptors and an L-isoaspartate-(D-aspartate) methyltransferase that recognizes covalently damaged proteins. Furthermore, the stability properties of these methylated proteins distinguish them from the products of this latter enzyme, which typically are hydrolyzed under conditions of SDS gel electrophoresis at pH 8.8 (Terwilliger & Clarke, 1981). The other two enzymes, the STE14 C-terminal isoprenylcysteine methyltransferase and the C-terminal leucine methyltransferase that is known to modify the catalytic subunit of protein phosphatase 2A (Xie & Clarke, 1993, 1994; Lee & Stock, 1993), have been described in yeast. The C-terminal leucine methyltransferase appears to be specific for the 36-kDa catalytic subunit of protein phosphatase 2A in mammalian cells and their 42–43-kDa homologs in yeast (see above). The 49- and 35-kDa yeast polypeptides are methylated in strains lacking the gene for the STE14 methyltransferase, clearly distinguishing the methyltransferase(s) for these polypeptides from the STE14 methyltransferase.

The chemical nature of the methyl ester linkage(s) in the 49- and 35-kDa polypeptides is not known. It is possible that they reflect the action of distinct types of L-glutamyl or C-terminal leucine methyltransferases or represent new sites of carboxyl methylation in proteins. We are presently interested in establishing the chemical nature of this linkage and in identifying the functional nature of the 49- and 35-kDa polypeptides.

Other Types of Protein Carboxyl Methylation Reactions in Yeast. The methylated species described here are those that incorporate detectable radioactivity under our experimental conditions. Methyl-accepting species present at very low concentrations, or where the methylation reaction is not stoichiometric, may not be detected. Furthermore, polypeptides that are only methylated under specific environmental situations would not be detected. For example, in intact cells, we detect very little methylation of the cytosolic 43-kDa species that is a major methyl acceptor when the cytosol is incubated directly with [^{3}H]AdoMet. As noted above, this 43-kDa species may correspond to the catalytic subunits of yeast protein phosphatase 2As. Its methylation may be dependent upon specific environmental conditions that can trigger a variety of intracellular responses (Engelberg et al., 1989).

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