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Methylation of Yeast Ribosomal Protein S2 is Elevated During Stationary Phase Growth Conditions

Daniel T. Lador^a, Brian L. Frey^a, Mark Scalf^a, Mark E. Levenstein^a, Jacklyn M. Artymiuk^a, and Lloyd M. Smith^{a,*}

Lloyd M. Smith: smith@chem.wisc.edu

^aDepartment of Chemistry, University of Wisconsin—Madison, 1101 University Avenue, Madison, WI 53706, USA

Abstract

Ribosomes, as the center of protein translation in the cell, require careful regulation via multiple pathways. While regulation of ribosomal synthesis and function has been widely studied on the transcriptional and translational “levels,” the biological roles of ribosomal post-translational modifications (PTMs) are largely not understood. Here, we explore this matter by using quantitative mass spectrometry to compare the prevalence of ribosomal methylation and acetylation for yeast in the log phase and the stationary phase of growth. We find that of the 27 modified peptides identified, two peptides experience statistically significant changes in abundance: a 1.9-fold decrease in methylation for k(Me)VSGFKDEVLETV of ribosomal protein S1B (RPS1B), and a 10-fold increase in dimethylation for r(DiMe)GGFGGR of ribosomal protein S2 (RPS2). While the biological role of RPS1B methylation has largely been unexplored, RPS2 methylation is a modification known to have a role in processing and export of ribosomal RNA. This suggests that yeast in the stationary phase increase methylation of RPS2 in order to regulate ribosomal synthesis. These results demonstrate the utility of mass spectrometry for quantifying dynamic changes in ribosomal PTMs.

Keywords

Ribosome; Post-translational Modifications; Mass Spectrometry; Proteomics; Stationary Phase; Growth Conditions

Introduction

Ribosomes are complex molecular machines consisting of about 79 proteins and 4 rRNA molecules (in *Saccharomyces cerevisiae*),¹ and they serve as the center of protein translation in the cell. Protein translation and the synthesis of new ribosomes are energy-intensive processes; ribosome synthesis utilizes 90% of cell energy in yeast during exponential growth.² Thus, it is important for the cell to carefully regulate ribosomal synthesis and

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Corresponding Author: Lloyd M. Smith, Department of Chemistry, University of Wisconsin-Madison. 1101 University Avenue, Madison, WI 53706. Telephone: 1-608-262-2594 Fax: 1-608-265-6780. smith@chem.wisc.edu.

Supplementary Material. Protein lists for representative log phase and stationary phase ribosome data are shown in the supplementary material.

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function. Ribosomal regulation has been found to be influenced by a number of environmental conditions, including nutrient availability, environmental stressors, and growth phase. Here, we examine regulation of ribosomes by using mass spectrometry to quantify the PTMs of yeast ribosomal proteins under different growth phase conditions.

The yeast population growth curve is generally characterized by three phases: the lag phase, the log phase, and the stationary phase.³ As yeast transition from log phase into stationary phase, the starvation conditions prompt the cells to enter a quiescent state, part of which involves regulating ribosomal synthesis and function.⁴ This regulation is an immensely complex process involving changes on multiple “levels” including: transcription,^{5–8} translation,⁹ small molecule¹⁰ and protein binding partners for the ribosome,¹¹ as well as post-translational modifications to ribosomal proteins.¹²

While the regulatory pathways at the transcriptional and translational levels have been widely explored, the role of PTMs of the ribosomal proteins has undergone substantially less investigation. Studies of the biological role of ribosomal protein PTMs generally involve creating binding assays or deletion mutants for enzymes known to modify other proteins in the cell, and then observing changes in ribosomal protein PTMs by gel electrophoresis and/or mass spectrometry.^{13–23} However, this approach requires prior knowledge of target enzymes and is limited to only identifying drastic PTM changes. One exception to this enzyme-focused approach is a study of phosphorylation in quiescent rat fibroblasts using a qualitative 2D-PAGE gel assay.¹² To our knowledge, there has yet to be a quantitative analysis of how ribosomal protein PTMs change under different environmental conditions. Here, we employ mass spectrometry to quantify the dynamic effect of growth phase on ribosomal protein methylation and acetylation.

Mass spectrometry is one of the foremost tools of the field of proteomics, which endeavors to identify all of the protein variants, or proteoforms, found in an organism.²⁴ Mass spectrometry is often coupled with on-line fractionation and pre-fractionation methods in order to lessen co-elution of analytes into the mass spectrometer, improving the number of peptides and proteins identified. Mass spectrometry is especially useful for elucidating the post-translational modifications of a protein, such as methylation, acetylation, and phosphorylation, which serve to alter or regulate the behavior of a protein. Mass spectrometry-based proteomics has been used previously to analyze ribosomal protein PTMs, although mostly by using the ribosome as a convenient model system for developing proteomics approaches that identify as many proteins and PTMs as possible.^{25–33} These proteomics papers have not focused on understanding the role of post-translational modifications on ribosomal proteins. Conversely, biological studies have delved into the role of specific enzymes or PTMs, but have not quantitatively monitored dynamic changes in protein modification. In this study, we combine these two approaches to quantify differences in PTM prevalence on yeast ribosomal proteins in the log phase of cell culture versus those in the stationary phase.

Materials and Methods

Yeast Growth

The YIT613 FLAG-tagged ribosome yeast strain used was a generous gift from Professor Toshifumi Inada at Nagoya University, Nagoya, Japan. The strain is engineered to express a FLAG tag (amino acid sequence DYKDDDDK) on ribosomal protein L25. YIT613 strain yeast was grown in YEPD media at 30°C, shaken at 200 RPM. Cultures (250 mL) were grown in the presence of 0.0045 g tetracycline and 0.0045 g chloramphenicol, and then transferred into 2 L cultures. Log phase yeast was harvested at OD₆₀₀ = 0.8, while stationary phase yeast was harvested after incubating for 48 hours. Yeast pellets were

washed with lysis buffer (20 mM Tris-HCl, 2 mM magnesium acetate, 100 mM potassium acetate, 100 μ g/mL cycloheximide), flash frozen with liquid nitrogen, and stored at -80°C . For this experiment, 3 biological replicates were grown to log phase, and 3 biological replicates were grown to stationary phase.

Ribosome Preparation

Purification of ribosomes was adapted from published reports.^{34, 35} One gram of wet yeast cells were resuspended in lysis buffer containing 10 μ L/mL HALT protease inhibitor (Thermo Scientific) and 425–600 μ m acid-washed glass beads (Sigma) and lysed with a Biospec Products Mini-Beadbeater-1. Cell debris was pelleted at 13,000 g for 5 minutes and the supernatant lysate was loaded onto a 2.5 mL column of ANTI-FLAG M2 Affinity Gel (Sigma). Bound ribosomes were washed with running buffer (50 mM Tris-HCl, 12 mM magnesium acetate, 100 mM ammonium chloride, 0.02% sodium azide, pH 7.4) and eluted with 100 μ g/mL FLAG peptide. Ribosomes were partially concentrated by vacuum evaporation and pelleted by ultracentrifugation using a Type 70 Ti rotor at 50,000 rpm (max 257,000 g) for 3 hours. Concentrated ribosome samples were stored at -80°C . This procedure was found to yield ~ 100 μ g of ribosomal protein by BCA assay.

Peptide Preparation

Ribosomal proteins were isolated using the acetic acid method.³⁶ Briefly, ribosomes were treated with 66% acetic acid and 0.1 M magnesium acetate for 2 hours and then centrifuged to pellet ribosomal RNA, after which ribosomal protein was recovered from the supernatant by precipitation with acetone. Figure 1 shows a representative SDS-PAGE analysis of the ribosomal protein preparation. Ribosomal proteins were dissolved in 8M urea, reduced with dithiothreitol, and alkylated with iodoacetamide, followed by a 7-fold dilution with 25 mM ammonium bicarbonate and digestion with trypsin overnight. Tryptic digests were desalted using Waters Sep-pak 1cc C18 cartridges, dried under vacuum, and dissolved in 0.1% formic acid.

LC-MS

All LC-MS experiments employed a capillary HPLC-ESI-MS/MS system consisting of a Waters nanoAcquity HPLC (Milford, MA) connected to an electrospray ionization (ESI) ion-trap/orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific, San Jose, CA). Analytical columns (15 cm) were prepared by packing 3 μ m-diameter 100 $\text{\AA}$ -pore C18 beads (Michrom Bioresources Inc., Auburn, CA) in 100 μ m ID $\times$ 365 μ m OD fused silica capillaries, with the capillary tip pulled to ~ 1 μ m with a P-2000 laser puller (Sutter Instruments, Novato, CA). For each sample, 0.15 μ g of ribosomal peptides were loaded onto the analytical column at 0.5 μ L/min for 30 min in an aqueous mobile phase containing 0.1% formic acid. Peptides were then eluted at 0.3 μ L/min with a gradient of increasing acetonitrile (2%–30% in the first 120 min, increasing to 70% in the next 6 min). A full-mass scan (300–1500 m/z) was performed in the orbitrap at a resolution of 60,000. The ten most intense peaks with $z > 1$ from the full scan were selected for fragmentation by either higher-energy collisional dissociation (HCD) or electron transfer dissociation (ETD) fragmentation. HCD employed 35% collision energy, while ETD employed 3.5×10^5 fluoranthene reagent ions and 5×10^4 precursor ions with a reaction time of 50 ms. The isolation width for the precursor ions was 2.5 m/z, and the product ions from fragmentation were analyzed in the orbitrap detector at a resolution of 7500. Dynamic exclusion was enabled with a repeat count of three over 30 s and an exclusion window of 120 s. Each biological replicate was run in triplicate for each fragmentation type (HCD & ETD), producing a total of 18 log phase ribosome runs, and 18 stationary phase ribosome runs.

Data Analysis

MS runs were analyzed individually with Thermo Proteome Discoverer 1.3. Peptides were searched against a *S. cerevisiae* database from Uniprot (downloaded 5/5/2011) with the following modifications: variable methionine oxidation (+15.995 Da), static carbamidomethylation of cysteines (+57.021 Da), variable lysine, arginine, and n-terminal methylation (+14.016) and dimethylation (+28.031), and variable lysine and n-terminal acetylation (+42.011). Mono-isotopic masses were used for both precursor and fragment ions with tolerances of 15 ppm and 0.02 Da, respectively. The searches allowed for up to two missed trypsin cleavages. The results were filtered using a 1% peptide false discovery rate. Methylated and acetylated ribosomal peptides were quantified in Xcalibur Qualbrowser using peak areas of the unfragmented peptide from extracted ion chromatograms (XIC) (+/-0.02 Da of the theoretical mass).

Peak areas were normalized using the total peak area of the entire total ion chromatogram (TIC). Peak area data were grouped by modified peptide and tested for significance with the student's t-test and the Benjamini-Hochberg correction.³⁷

Results

The experimental workflow was designed to produce highly pure samples with minimal steps (Figure 2). Purity of ribosomal proteins prior to tryptic digestion was demonstrated by the SDS-PAGE gel shown in Figure 1, which matches similar separations of pure ribosomal protein samples as seen in the literature.³⁶ This coincides with the proteomics results, which demonstrated sample purity on the peptide level. A representative LC-MS experiment on log phase ribosomes detected 96 ribosomal proteins, with 93% of the 7991 peptide spectral matches (PSMs) corresponding to yeast ribosomal peptides; for stationary phase ribosomes, we detected 98 ribosomal proteins with 90% of the 8615 PSMs corresponding to ribosomal peptides (Supplementary Material). PSMs refer to every MS/MS spectrum that can be matched to a peptide, making PSMs the best gauge of sample purity in a quantitative LC-MS study. This high quality for both the log phase and stationary phase ribosomes is invaluable for the present quantitative comparison study.

Sample purity was also important for optimizing the number of PTMs observed. On average, LC-MS analysis of the FLAG-tag affinity purified ribosomes detected 20 methylated or acetylated peptides per experiment, with 27 modified peptides detected across several replicate experiments (Table 1). While ribosomes are regularly seen in proteomic analyses of full yeast digests, a search for methylated and acetylated ribosomal peptides in a full yeast digest experiment yielded only 8 modified peptides on average (data not shown). Pre-fractionating yeast digest peptides into eight fractions using off-line high-pH reverse phase chromatography was found to improve the number of methylated or acetylated peptides identified to 24, at the cost of eight times the instrument analysis time. Thus, utilizing FLAG-tag affinity purification allowed us to improve the quality of our data while shortening required instrument time, allowing for analysis of multiple biological and technical replicates.

To further expand the range of peptides detected, we analyzed each sample using both higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) fragmentation, which have been found to produce complementary data.³⁸ As shown in Table 1, eleven modified peptides were only detected using HCD, while three peptides were detected only using ETD. Thirteen peptides were detected using both methods. Thus, while ETD fragmentation allowed us to detect a few additional modified peptides, it may not be worth the additional instrument time in future experiments.

Quantitative data showing the relative amounts of modified ribosomal peptides in log phase versus stationary phase yeast is also shown in Table 1. Modified peptides were quantified by constructing the XIC of the most intense isotopic peak in the full scan and then measuring the peak area (Figure 3). While spectral counting is commonly used for label-free quantification of proteins, our goal was to quantify PTM prevalence on the peptide level. Furthermore, label-free quantification by peak area has been shown to be a useful tool for analyzing complex samples.³⁹ Peak areas were normalized using the TIC peak area to correct for differences in injected sample amount. When comparing the log phase to the stationary phase, some PTMs were seen to increase in abundance, while others decreased. Most changes were minor, ranging between a 1.5-fold decrease and a 1.5-fold increase, but occasionally larger changes were observed, including a 10-fold increase for the dimethylated rGGFGGR peptide in the stationary phase.

Statistically significant changes in modified peptide quantity between log phase and stationary phase samples were found using the student's t-test⁴⁰ with the Benjamini-Hochberg correction.³⁷ Two peptides met the p-value cutoff of 0.05: a 1.9-fold decrease in methylation for k(Me)VSGFKDEVLETV of RPS1B, and a 10-fold increase in dimethylation for r(DiMe)GGFGGR of RPS2. We will henceforth refer to these modifications as RPS1B K243 methylation and RPS2 R10 dimethylation, which indicate the protein and the amino acid position of the PTM. While we did not find published reports in the literature on the biological role of RPS1B K243 methylation, RPS2 R10 dimethylation is a known modification, and we discuss below its biological roles.

Discussion

It is interesting to find two cases where methylation depends on growth phase, as methylation has been a recent focus of studies of ribosome regulation.^{13, 15, 16, 18–23} RPS2 R10 dimethylation is of particular note, in that it is 10-fold more abundant in the stationary phase. Lipson et al. informally observed that RPS2 methylation varied depending on growth conditions, although they did not publish this data.²²

Methyltransferase studies have identified Rmt3 in *S. pombe*, Rmt1 in *S. cerevisiae*, and Prmt3 in mouse as catalysts for RPS2 R10 methylation, but gene deletion studies of these three enzymes have found differing phenotypic results. *S. pombe* Rmt3 was found to exhibit a methyltransferase-independent function that plays a role in regulating the production of the 40S subunit.^{20, 41–43} In mammalian cells, mice lacking Prmt3 exhibit smaller embryos during gestation, but ribosome stability and function are not affected.^{21, 44} Lastly, deletion of Rmt1 in *S. cerevisiae* causes no phenotypic difference in ribosome stability.

However, Rmt1 is known to bind and/or methylate a number of proteins that process and export mRNA; methylation of Rmt1 target proteins Npl3, Hrp1, and Nab2 promotes their dissociation from nuclear factors and facilitates their export from the nucleus.^{46–48} This is consistent with RPS2's known functions of processing and exporting 20S rRNA, a precursor for 40S ribosomal subunits.^{42, 45} Our observation that RPS2 R10 dimethylation increases tenfold in stationary phase suggests that the cell may be facilitating export of RPS2 from the nucleus. This would result in slowing processing and export of 20S rRNA, which in turn restricts 40S ribosomal subunit production, a known response to the depletion of available nutrients. Therefore, methylation of RPS2 may play a role in regulating export of rRNA to the cytosol.^{22, 23}

Our observation of RPS2 R10 dimethylation increasing 10-fold in stationary phase yeast provides new evidence for the role of RPS2 methylation in 20S rRNA processing and export. To our knowledge, the observation of RPS1B K243 methylation decreasing in

stationary phase yeast has not been reported, suggesting a novel role for RPS1B methylation that invites further investigation. This work exhibits the utility of mass spectrometry for quantifying dynamic changes in PTMs without prior knowledge of target enzymes and validates mass spectrometry as a valuable tool for investigating the biological role of PTMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PTM	post-translational modification
RPS1B	ribosomal protein S1B
RPS2	ribosomal protein S2
BCA	bicinchoninic acid
HPLC	high-performance liquid chromatography
ESI	electrospray ionization
m/z	mass-to-charge ratio
HCD	higher-energy collisional dissociation
ETD	electron transfer dissociation
PSM	peptide spectral match
XIC	extracted ion chromatogram
TIC	total ion chromatogram

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Highlights

- Ribosomal protein modifications were quantified for yeast in different phases of growth.
- Of 27 confidently identified methylated and acetylated peptides, two change significantly.
- Methylated ribosomal protein (RP) S1B decreases 1.9-fold, inviting further investigation.
- Dimethylated RP S2 increases 10-fold, suggesting a role in regulating ribosome synthesis.
- Mass spectrometry is useful for investigating the role of post-translational modifications.

Figure 1. SDS-PAGE gel of *Saccharomyces cerevisiae* ribosomal proteins. Lane A contains molecular weight markers. Lane B contains ribosomal proteins after acetic acid wash, acetone precipitation, and resuspension in SDS-PAGE buffer. Multiple proteins are visible, ranging from 5 kDa to 45 kDa.

Figure 2.

Experiment workflow. Three replicates each of YIT613 strain yeast were grown to log phase and stationary phase. Yeast were lysed by bead beating and loaded onto an ANTI-FLAG column. FLAG-tagged ribosomes were retained on the column and eluted with FLAG peptide. Ribosomal RNA was precipitated in 66% acetic acid and 0.1M magnesium acetate and dissolved ribosomal proteins were collected by acetone precipitation. Ribosomal proteins were resuspended in 8M urea and digested with the proteolytic enzyme trypsin, and the resulting tryptic peptides were analyzed by LC-MS.

Figure 3.

A representative LC-MS experiment showing data for dimethylated peptide rGGFGGR. In Fig. 3A, the TIC represents all of the yeast ribosomal peptides detected by the mass spectrometer. Several full scan mass spectra are taken per second, each of which may contain peaks from multiple peptides. Fig. 3B shows the isotopic distribution of the +2 charge state for rGGFGGR as seen in a full scan mass spectrum. In Fig. 3C, the XIC corresponds to the signal intensity over time of the 367.71 m/z ion, the most intense isotopic peak for rGGFGGR. The peak area is integrated to yield a quantitative measure of the amount of peptide in the sample. Finally in Fig. 3D, the 367.71 m/z ion was isolated for HCD fragmentation and the MS/MS spectrum is shown. The peptide is identified by comparison to a Uniprot *S. cerevisiae* database, and characteristic fragmentation peaks are checked for mass shifts to identify PTMs. A mass shift of +42.01 indicates the presence of an acetyl group, while a +14.02 or +28.03 mass shift corresponds to a methyl or dimethyl group, respectively. Trimethylations (+42.04) were not detected.

Table 1

Methylated and acetylated ribosomal peptides detected by mass spectrometry. Peptides were fragmented with HCD and/or ETD fragmentation, and then searched against a Uniprot *S. cerevisiae* database. Twenty-seven modified peptides were identified and quantified XIC peak area across all biological and technical replicates. The average peak areas were used to find the stationary phase/log phase ratio, and tested for statistical significance with the student's t-test. Resulting p-values were adjusted with the Benjamini-Hochberg correction, and two peptides, rGGFGGR and kVSGFKDEVLETV were found to exhibit statistically significant changes in modified peptide quantity.

Protein	Peptide Sequence	Modifications	Fragment	Ratio	P Value	Adj. P Value
S2	rGGFGGR	R1(Dimethyl)	HCD/ETD	10.54621	0.0003	0.00954
S1-B	kVSGFKDEVLETV	K1(Methyl)	HCD/ETD	0.532633	0.0005	0.00726
L7-A	TAEQVAAEr	R9(Methyl)	HCD	0.752925	0.0069	0.06706
S1-B	VSGFRDEVLETV	K5(Methyl)	HCD	0.738775	0.0507	0.36743
L8-A	KMGVPYAIVk	K10(Methyl)	HCD/ETD	0.782643	0.0569	0.32985
L12	IQNtQAAASVVPSSSLVITALK	R4(Methyl)	HCD/ETD	1.407789	0.0805	0.38913
S21-A	AIPGEYVTYALSGYYr	R16(Methyl)	HCD/ETD	0.71243	0.0982	0.40684
L9-A	YIQTEQQIEVPEGVTVSik	K19(Methyl)	HCD	1.783777	0.1166	0.42264
L33-A	IEGVATPQDAQFYLGk	K16(Methyl)	HCD/ETD	0.825141	0.1212	0.39053
S29-B	VCSSHTGIVrK	C2(Carbamidomethyl); R10(Methyl)	HCD	0.537515	0.1282	0.37184
S21-A+B	MENDkGQLVELYVPR	K5(Acetyl)	HCD/ETD	0.785795	0.153	0.40337
L42	KQSGFGGQTrPVFHK	K10(Methyl)	HCD/ETD	0.698218	0.1846	0.44621
L33-A	IEGVATPQDAQFYLGkR	K16(Methyl)	HCD	1.306633	0.2736	0.61041
L42	KQSGFGGQTrPVFHKK	K10(Methyl)	ETD	0.645514	0.2845	0.54997
L23	DGVFLYFEDNAGVIANPKGEMkGSAITGPVGK	K18(Dimethyl); K22(Dimethyl)	HCD	2.597351	0.3172	0.57495
L42	QSGFGGQTrPVFHKK	K9(Methyl)	HCD/ETD	0.660266	0.3593	0.61289
L31-A	LHMGTDVr	R9(Methyl)	HCD	0.901475	0.382	0.61539
S8	KNVKEETVAK	K4(Acetyl)	ETD	0.64605	0.3997	0.61007
L42	QSGFGGQTrPVFHK	K9(Methyl)	HCD/ETD	0.696164	0.4622	0.67022
L24-B	MkVEVDSESGAK	K2(Methyl)	HCD/ETD	0.890899	0.5313	0.73371
L16-A	LkVFEGIPPPYDK	K2(Methyl)	ETD	0.905814	0.5475	0.72173
L16-A	LkVFEGIPPPYDKK	K2(Methyl)	HCD/ETD	0.909256	0.6444	0.77869
L42	ASLFAQGR	K8(Methyl)	HCD/ETD	0.862425	0.802	0.93037

Protein	Peptide Sequence	Modifications	Fragment	Ratio	P Value	Adj. P Value
S21-A+B	MENDKGGQLVELYVPRK	M1(Oxidation); K5(Acetyl)	HCD	0.904987	0.8059	0.89888
S29-B	VCSSHTGIVr	C2(Carbamidomethyl); R10(Methyl)	HCD	0.928612	0.8303	0.89179
S28-A	MDNKTPVTLAK	K4(Acetyl)	HCD	1.013954	0.9499	0.98383
S21-A+B	MENDKGGQLVELYVPRK	K5(Acetyl)	HCD	0.997643	0.9786	0.97858