Rapid Cytoplasmic Turnover of Yeast Ribosomes in Response to Rapamycin Inhibition of TOR

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The target of rapamycin (TOR) pathway is the central regulator of cell growth in eukaryotes. Inhibition of TOR by rapamycin elicits changes in translation attributed mainly to altered translation initiation and repression of the synthesis of new ribosomes. Using quantitative analysis of rRNA, we found that the number of existing ribosomes present in a *Saccharomyces cerevisiae* culture during growth in rich medium rapidly decreases by 40 to 60% when the cells are treated with rapamycin. This process is not appreciably affected by a suppression of autophagy, previously implicated in degradation of ribosomes in eukaryotes upon starvation. Yeast cells deficient in the exosome function or lacking its cytoplasmic Ski cofactors show an abnormal pattern of rRNA degradation, particularly in the large ribosomal subunit, and accumulate rRNA fragments after rapamycin treatment and during diauxic shift. The exosome and Ski proteins are thus important for processing of rRNA decay intermediates, although they are probably not responsible for initiating rRNA decay. The role of cytoplasmic nucleases in rapamycin-induced rRNA degradation suggests mechanistic parallels of this process to nutrient-controlled ribosome turnover in prokaryotes. We propose that ribosome content is regulated dynamically in eukaryotes by TOR through both ribosome synthesis and the cytoplasmic turnover of mature ribosomes.

In eukaryotic organisms from yeasts to humans, the conserved TOR signaling pathway plays the central role in regulating cellular responses to nutrient availability and mitogenic signals (4, 65, 67). Rapamycin is a potent TOR inhibitor that inhibits cell proliferation and growth (6, 68) and induces a number of coordinated changes in gene expression characteristic of starvation conditions (13, 24). Rapamycin and related compounds (52) find clinical use owing to their antiproliferative, immunosuppressive, and antitumor properties (39, 59) and have also attracted attention for their ability to increase life span in a range of species (25, 44). Our understanding of the molecular basis for these complex physiological responses would clearly benefit from knowledge of the full spectrum of events occurring in cells after TOR inactivation.

An essential part of the TOR-mediated control of cell growth and proliferation is effected at the level of translation and involves translation initiation (6, 16) and biosynthesis of new ribosomes (45). The ribosome content dictates the cell's translational capacity and has long been known to correlate with the nutrient-controlled rate of exponential growth in microorganisms (29, 36, 51). Ribosome synthesis in *Saccharomyces cerevisiae* is activated in response to favorable growth conditions, largely through TOR-regulated increases in rRNA transcription and the expression of genes encoding ribosomal proteins and assembly factors, termed the *RP* and *Ribi* regulons (4, 27, 38, 65). Conversely, inactivation of TOR causes repression of *RP* and *Ribi* gene expression and inhibits transcription and maturation of rRNAs (13, 26, 45, 46, 68), thereby causing an effective shutdown of ribosome biogenesis.

In this study, we present evidence to suggest that TOR control of the cellular ribosome content extends beyond the regulation of new ribosome synthesis. Our data indicate that inactivation of TOR with rapamycin in growing yeast triggers a large-scale reduction of the ribosome content through the rapidly occurring turnover of the existing ribosomes. This process involves cytoplasmic nucleases and appears to be mechanistically distinct from the previously described vacuolar degradation of ribosomes (32, 57) and

nonfunctional rRNA decay (NRD) (17, 22, 34). These findings reveal a new layer in the mechanisms through which TOR controls the translational capacity of a eukaryotic cell.

MATERIALS AND METHODS

Yeast culture. *S. cerevisiae* cells were cultured at 30°C with shaking in YPD (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 100 mg/liter adenine or in synthetic dextrose (SD) medium prepared with complete supplement mixture (CSM) amino acid supplements (Sunrise Science). Rapamycin (Calbiochem) was used at the final concentration of 100 nM, added from a 10 μ M stock solution prepared in 99% ethanol–1% Tween 20. Cycloheximide (Sigma) was used at 25 μ g/ml. New strains (see Table S1 in the supplemental material for strain descriptions) were generated using standard gene replacement techniques. Correct disruption of the targeted loci was confirmed by PCR for all strains used in this study.

For one set of experiments (see Fig. 1), overnight cultures in SD were diluted ~10-fold with fresh medium, grown at 30°C with shaking for 4 h to an optical density at 600 nm (OD_{600}) of 0.6 to 0.7 ($\sim 6 \times 10^6$ cells/ml), and divided into two parts. Rapamycin (100 nM) was added to one portion of the culture, which continued to be incubated in a constant volume. The other part of the culture was maintained in a steady-growth state. To prevent depletion of nutrients, the OD_{600} was determined hourly and the culture was diluted as appropriate with prewarmed medium to maintain an OD_{600} between 0.85 and 1.35. The volume of the added medium was recorded to calculate the effective total volume of the culture at each sampling point.

For the analysis of individual mutant strains, cells were grown in YPD overnight, diluted to an OD_{600} of 0.15, and grown to an OD_{600} of 0.6 to 0.8

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(reached after ~4 h). After that, rapamycin was added and incubation continued in a constant volume. For some experiments (see Fig. 3B and C, 4A, and 5A), either rapamycin or the same volume of vehicle was added to cells.

Determination of the rRNA content. The framework of each ribosomal subunit consists of large rRNA molecules (25S rRNA in the yeast 60S ribosomal subunit and 18S rRNA in the 40S subunit), which can be detected with high specificity by hybridizations with radiolabeled oligonucleotide probes. Since the mature full-length rRNAs are integral components of ribosomal subunits and every subunit has a single region of complementarity to each probe, hybridization analysis of rRNA done in a quantitative manner can be used to monitor changes in the number of ribosomes present in a culture. Furthermore, the total ribosome content of a culture grown in liquid can be determined based on the amount of rRNA present in a small sample withdrawn from the culture, as long as the sample volume and the total culture volume are known. With these considerations in mind, we devised an approach in which we extracted RNA from precisely measured volumes of a yeast culture, separated rRNAs using gel electrophoresis, and quantified 25S and 18S rRNAs by Northern hybridizations with specific oligonucleotide probes (probe sequences are provided in Table S2 in the supplemental material). To reduce variability, samples withdrawn from a culture at different time points were precipitated and immediately frozen at -80°C . RNA extraction with hot acid phenol (49) was carried out in parallel for the entire sample set.

The total rRNA content (R_t), which reflects the number of ribosomes present in a culture, was defined as follows: $R_t = R_s \times (V_t/V_s)$, where V_t is the total culture volume and R_s is the rRNA amount in a sample of the volume V_s . Because we were interested in relative R_t changes rather than absolute ribosome numbers, we used phosphorimager-derived values (the total hybridization signal within blot areas containing rRNA bands) obtained from hybridization of the same membrane for calculations with a matching set of samples (e.g., wild-type [WT] and mutant strains before and after rapamycin treatment). Control experiments were used to establish the working range in which the probe hybridization signal was a linear function of the RNA amount loaded onto the gel. Nonlinear regression analysis of the data was performed with Prism 5 (GraphPad Software), assuming constant rates of growth and one-phase decay. At least two independent cultures were used in all quantitative measurements of RNA.

Analysis of labeled rRNA. To evaluate the rRNA turnover rate in a growing yeast culture, rRNA was prelabeled in W303 cells diluted in SD to an OD_{600} of ~0.3 by growing the cells in the presence of $10 \mu\text{Ci/ml}$ [$5,6\text{-}^3\text{H}$]uracil (Perkin Elmer) for 4 h. During labeling, the concentration of cold uracil in SD was lowered from 20 to 5 mg/liter, which did not appreciably affect cell growth. After labeling, cells were pelleted, washed with regular SD, resuspended in fresh medium, and incubated for a further 1.5 h, which was necessary to allow cells to resume growth at a constant rate. Additionally, after this time, all label is incorporated into mature rRNA and labeled rRNA precursors are no longer observed. Cells were diluted hourly with prewarmed medium as described above. Rapamycin was added when indicated at 30 min after labeling, and cells were maintained in a constant volume. To calculate the total amount of labeled rRNA per culture, we used the same approach used for the determination of the total rRNA content, but instead of performing hybridizations, we visualized 25S and 18S rRNA bands after their transfer onto a membrane by staining with methylene blue, the bands were excised from the membrane and placed into scintillation vials, RNA was hydrolyzed in $200 \mu\text{l}$ of 0.25 M NaOH for 3 h at 42°C , and the ^3H activity was measured by scintillation counting.

Viability and autophagy assays. Equal volumes were taken from four independent cultures of each strain before and after rapamycin treatment, diluted to obtain between 10^2 and 10^3 colonies per plate, and spread onto YPD plates. The number of viable cells was determined by counting colonies appearing on day 3 after plating (day 4 for rapamycin-treated W303 cells, which recover more slowly from rapamycin). To monitor macroautophagy, strains were transformed with a green fluorescent protein

(GFP)-Atg8 expression construct and analyzed as described previously (53).

RESULTS

Rapamycin treatment triggers a rapid decrease of the ribosome content. Previous studies found that the synthesis of new ribosomes stops within minutes after treatment of yeast cells with rapamycin (45, 46). To investigate the fate of ribosomes remaining in cells after TOR inactivation, we performed quantitative assays to examine the rRNA contents in the common laboratory *S. cerevisiae* strain W303 before and after rapamycin treatment. We quantified 25S and 18S rRNAs in precisely measured culture samples by Northern hybridizations with specific oligonucleotide probes (see Materials and Methods for a detailed description). For validation of this approach, we first analyzed rRNA during steady-state growth, which is known to result in a proportional increase in all cellular components (29, 62). Measurements of optical density were used to confirm that the culture grew exponentially and calculate its doubling time (T_d) (Fig. 1A). The total content of 25S and 18S rRNAs was experimentally determined from rRNA hybridizations (Fig. 1B, lanes 8 to 12), and the derived T_d for rRNAs (2.07 to 2.10 h) (Fig. 1C) was found to correspond within 5% to the expected value based on the T_d for cells (2.00 h) (Fig. 1A), indicating that this method provides an accurate measure of the rRNA content.

In agreement with results from previous studies, the addition of rapamycin suppressed growth in a yeast culture (Fig. 1A). Surprisingly, the rRNA content after rapamycin treatment did not remain steady, as would be expected if the existing ribosomes were stable, but instead declined (Fig. 1B, lanes 1 to 7). Turnover of 25S and 18S rRNAs occurred mostly within the first 5 to 6 h and could be approximated by one-phase decay (Fig. 1D). The rRNA levels eventually stabilized and remained steady for at least 20 h (Fig. 1B, lanes 6 and 7).

The drop in the rRNA content in cells exposed to rapamycin could be due to two possibilities, although these are not mutually exclusive. First, the degradation rate of ribosomes may be substantially increased after rapamycin treatment compared to that in cells in the previous state of active growth. Second, ribosome degradation may occur in growing cells at a similar rate but be masked by the influx of new ribosomes and become apparent only when ribosome synthesis is stopped by TOR inactivation. To address these possibilities, we repeated the analysis described above with cells in which rRNA was prelabeled with [^3H]uracil and calculated the total amount of labeled 25S and 18S rRNAs present in the culture between 1 and 5 h after labeling. In exponentially growing cells, no detectable change in the labeled rRNAs was observed during this time (Fig. 1E), indicating that ribosomes were stable under these conditions. In contrast, the amount of labeled rRNAs decreased dramatically when cells were treated with rapamycin (Fig. 1F). The high ribosome stability observed in exponentially growing cells is in agreement with the findings of previous studies of a variety of microorganisms during steady growth (18, 29, 34, 43). We conclude that the rate of ribosome turnover is greatly increased by rapamycin compared to that in cells in an exponentially growing culture.

As shown below, the effects of rapamycin on ribosome stability are reproducibly observed in different strains of *S. cerevisiae*, although the kinetics of rRNA turnover can be influenced by the strain background and growth medium to some extent (Fig. 2A).

FIG 1 Large-scale rRNA turnover takes place in rapamycin-treated yeast. (A) Growth curves of W303 cells in SD medium, untreated or treated with rapamycin (Rap). Symbols represent individual OD₆₀₀ measurements for duplicate cultures. OD₆₀₀ values for untreated cells were fit to the exponential growth equation by nonlinear regression analysis; the number of samples (n) and the R^2 value are shown to indicate the quality of the fit. (B) Analysis of rRNA from cultures represented in panel A. RNA from precisely measured culture volumes corresponding to a defined fraction of a culture, as indicated for each lane, was separated on a gel; fraction size was reduced for untreated cells to avoid overloading the gel. The RNA was blotted, stained with methylene blue, and hybridized with ³²P-labeled probes to quantify 25S and 18S rRNA levels. (C) Total rRNA content under steady growth conditions. Symbols show the average percentage for two cultures relative to the RNA content measured at 0 h; error bars display ranges. A regression analysis was performed as described for panel A. (D) Total rRNA content in rapamycin-treated cultures. Results are displayed as described for panel C, but data were fit to a one-phase decay model. $T_{1/2}$, half-life. (E) Total amount of rRNA prelabeled with [³H]uracil in an exponentially growing culture. See Materials and Methods for details. Data from two independently grown W303 cultures are shown. The constant rate of growth was verified by monitoring the OD₆₀₀ as described in the legend to panel A. (F) Data are as described for panel E except that cells were treated with rapamycin at time zero.

In principle, a drop in rRNA levels could be explained by degradation in dead cells; however, a viability assay ruled out this scenario (Fig. 2B). To control for nonspecific effects of rapamycin, we assayed rRNA from cells lacking the prolyl isomerase Fpr1, which binds rapamycin and is required for TOR inhibition by this drug in yeast (31, 35). The *fpr1Δ* cells showed no evidence of rRNA turnover (Fig. 2C and D), consistent with the idea that the rapamycin-induced decrease in the rRNA content is due to TOR inhibition rather than a side effect of the drug.

Role of autophagy in rapamycin-induced degradation of ribosomes. The finding that TOR inactivation causes turnover of a large fraction of ribosomes led us to explore the mechanisms un-

derlying this phenomenon. Rapamycin treatment mimics many aspects of starvation and activates catabolic processes in cells, including autophagy (41). Microscopically, ribosomes were observed in autophagic bodies appearing in vacuoles during nonselective macroautophagy (57) and the piecemeal microautophagy of the nucleus (PMN) (47). From studies of ribosomal proteins appearing in the yeast vacuole upon starvation, a specialized autophagic pathway termed ribophagy was also recently proposed to mediate ribosome degradation (32). Given these data, we asked whether autophagy could explain the large-scale ribosome turnover taking place after rapamycin treatment. If ribosomes are eliminated through autophagic pathways, inhib-

FIG 2 Rapamycin-induced decrease of rRNA does not correlate with a loss of viability and requires Fpr1, the cellular rapamycin cofactor for TOR inhibition. (A) W303 and BY4741 cultures were grown in SD to an OD_{600} of 0.7 to 0.8 and treated with rapamycin. Symbols show the average total rRNA contents in duplicate cultures, normalized to the average in each strain at 0 h; error bars indicate ranges. (B) The numbers of CFU determined for quadruplicate cultures of the strains represented in panel A did not change significantly after rapamycin treatment ($P = 0.63$ for W303; $P = 0.13$ for BY4741 [Wilcoxon signed-rank test]). Horizontal lines denote median values. (C) Hybridization analysis of rRNA in *fpr1Δ* and isogenic WT strains before and after rapamycin treatment. (D) Quantitative analysis of the hybridization data demonstrates that rRNA continues to increase in cells rendered rapamycin insensitive by the *fpr1Δ* deletion. The average values for two independent cultures are shown; error bars indicate ranges.

iting autophagy would be expected to stabilize rRNA under these conditions.

We first analyzed cells lacking the *ATG1* gene, which encodes a protein kinase essential for autophagy in yeast (37). To confirm suppression of autophagy, we used an established procedure to monitor cleavage of the GFP-Atg8 marker protein (15) expressed in the cells (Fig. 3A). Quantitative analysis performed in two different strain backgrounds showed that following rapamycin treatment, the decreases in rRNA in *atg1Δ* cells and autophagy-competent WT cells were similar (Fig. 3B and C). We next examined strains lacking other proteins implicated in targeting ribosomes to the vacuole: Atg7, an E1-like enzyme required for most types of autophagy in yeast (30, 58), Ubp3 and Bre5, involved in ribophagy (32), Nvj1, a nuclear envelope protein mediating PMN (42), and Atg18, involved in multiple autophagy types, including the cytoplasm-to-vacuole targeting (Cvt) pathway and PMN (7, 23, 33). In all tested deletion strains, the rRNA levels measured 5 h after rapamycin treatment were similar to those in the isogenic WT cells (Fig. 3D), indicating that previously studied autophagy-dependent mechanisms do not contribute significantly to the rapid decrease in the ribosome content after rapamycin treatment.

The exosome participates in 25S rRNA turnover in rapamycin-treated cells. Because the above-described results suggested that ribosome degradation may not require autophagic targeting to the vacuole, we considered the possibility that this process might involve cytoplasmic enzymes. We first investigated the effects of rapamycin in cells deficient in the function of the exosome, a multiprotein complex that provides essential exo- and endonu-

lease activities for processing and decay of RNA in eukaryotes (48). We examined two strains in which the core exosome subunit Rrp41 (40) or the core-associated 3' exonuclease Dis3/Rrp44 (20) was expressed from a Tet-regulated promoter. When expression of either Rrp41 or Dis3 was turned off by the addition of doxycycline for 5 h, rapamycin treatment induced marked accumulation of rRNA decay products, especially noticeable for 25S rRNA (Fig. 4A, compare Dox 5 h lanes), suggesting that their efficient digestion requires the exosome. In addition, there was accumulation of a characteristic large 25S rRNA decay intermediate [$*25S(3')$] (Fig. 4A), observed previously in cells in which loss of function of the essential ubiquitin ligase Rsp5 leads to nucleolytic degradation of ribosomes (53).

Notably, long-term depletion of Rrp41 or Dis3 increased 25S rRNA decay products even in the absence of rapamycin (Fig. 4A, Dox 16 h lanes), suggesting ongoing low-level rRNA decay and/or destabilizing effects on ribosomes caused by the lack of exosome function. In order to better understand the difference between these effects and those caused by rapamycin, we incubated cells expressing Rrp41 from a Tet-regulated promoter (designated *Tet-RRP41* cells) with doxycycline for 5 h, split the culture into two parts, added rapamycin to one part, and monitored the total amounts of rRNAs and their degradation products in both rapamycin-treated and untreated cells for the next 6 h (Fig. 4B). Cells in which the expression of Rrp41 was shut off were impaired in growth and their rRNA content increased less than 1.5-fold during this time (Fig. 4B, -Rap), consistent with the exosome requirement for ribosome biogenesis (1, 40). In the presence of rapamycin,

FIG 3 Analysis of rapamycin-induced ribosome turnover in autophagy-deficient strains. (A) Results from a GFP-Atg8 processing assay to confirm inhibition of autophagy in *atg1Δ* strains. Cells transformed with a GFP-Atg8p plasmid were analyzed by immunoblotting of total cell lysates with anti-GFP antibodies. As expected, deletion of *ATG1* in the W303 strain abolished GFP-Atg8 processing, readily observed in WT cells, after depletion of nutrients from the medium (lanes 1 and 2). A weak band of free GFP, however, was detectable in W303 *atg1Δ* cells after a short rapamycin treatment (lanes 5 and 6), due presumably to susceptibility of the fusion protein to proteolysis in this strain. We therefore created an *atg1Δ* strain in another genetic background (MK1). Atg8-GFP cleavage was undetectable in this strain after rapamycin treatment (lanes 7 and 8), as well as in saturated cultures (lanes 3 and 4), thus providing a clear indication of the lack of macroautophagy. (B) Northern hybridizations of 25S rRNA (with probe y503) and 18S rRNA (with probe y500) isolated from equal culture volumes of the *atg1Δ* and isogenic WT strains, with or without rapamycin treatment. The strain background is indicated to the left. (C) Quantification of the rRNAs in strains represented in panel B. Total rRNA content at each sampling point was normalized to the average content in the same strain at 0 h. (D) rRNA remaining in the indicated strains after a 5-h rapamycin treatment. All results are the average for two independent cultures; error bars indicate ranges.

cin, the total amount of 25S and 18S rRNAs decreased (Fig. 4B, +Rap). When we compared the amount of rRNA degradation products detectable as a smear below the full-length rRNAs on a gel (Fig. 4A) to the full-length rRNA levels, we found that accumulation of 25S, but not 18S, decay intermediates was significantly accelerated in Rrp41-deficient cells by rapamycin (Fig. 4C). This result suggests that degradation routes for 25S and 18S rRNAs in rapamycin-treated cells are nonidentical and that the exosome is important for 25S rRNA decay.

Collectively, these data indicate that the exosome contributes to the efficient processing of 25S rRNA degradation intermediates in yeast cells. Even short-term exosome depletion leads to an increased level of 25S rRNA decay products during rapamycin treatment. Given that rRNA constitutes ~85% of total RNA in rapidly growing yeast (62) and about half of rRNA is turned over after rapamycin treatment (Fig. 1D), a mild exosome deficiency may be sufficient to promote the accumulation of decay products under these conditions.

Cytoplasmic exosome cofactors Ski7 and the SKI complex are required for efficient 25S rRNA degradation. The cytoplasmic exosome is associated with the G-protein Ski7 and the SKI complex, composed of Ski2, Ski3, and Ski8, which were shown previously to provide substrate targeting and regulatory functions during degradation of mRNA and viral RNAs (2, 9, 21, 60, 61). Rapamycin treatment of *skiΔ* strains caused pronounced accumulation of the *25S(3') species and smearing of heterogeneous decay fragments of 25S rRNA (Fig. 5A), similar to the effects in cells

with depletion of Rrp41 and Dis3 (Fig. 4A). Interestingly, low levels of *25S(3') RNA were detectable in various *skiΔ* mutants even under normal growth conditions (Fig. 5A, –Rap lanes, and B, Rap 0 h lanes). Deletions of *SKI2*, *SKI3*, *SKI7*, and *SKI8* gave identical results, consistent with the formation of a stable Ski2-Ski3-Ski8 complex (12) that interacts with Ski7 (3). Quantitative analyses of *ski2Δ* and *ski7Δ* strains showed gradual accumulation of degradation products of 25S rRNA relative to intact rRNA upon rapamycin treatment (Fig. 5B and C). Deletion of *SKI2* and *SKI7* did not have a significant effect on the full-length rRNA decay rates (Fig. 5D), consistent with a defect at intermediate rather than initial steps of 25S rRNA degradation. The role of Ski7 and the SKI complex in 25S rRNA degradation may thus be similar to that in no-go decay (NGD) of mRNA, in which they are thought to recruit exonucleases after an initializing event, presumably carried out by endonucleases (19). The overall similarities to Rrp41 and Dis3 depletion (Fig. 4A) provide strong indication that the SKI complex and Ski7 act in conjunction with the exosome to promote the cytoplasmic decay of 25S rRNA.

Decay of 25S rRNA upon nutrient depletion utilizes the Ski-exosome system, is distinct from NRD, and requires ongoing translation. We next asked whether the above-described findings are relevant for ribosome turnover occurring in a yeast culture during natural nutrient depletion (28) and not just in rapamycin-treated cells. We analyzed rRNA in cells grown in YPD for 48 h, which results in the diauxic shift following glucose depletion from the medium (66). Consistent with defects in 25S rRNA turnover

FIG 4 Effects of exosome depletion on rRNA decay. (A) rRNA decay intermediates accumulate after depletion of the core exosome subunit Rrp41 or the core-associated nuclease Dis3. Doxycycline (Dox) was added to switch off expression of Rrp41 or Dis3 for the indicated times before cells were treated, or left untreated, with rapamycin (Rap) for 5 h; the WT strain is R1158. Equal total RNA samples were loaded into the lanes, and blot exposure was increased to better visualize rRNA decay products. (B) *Tet-RRP41* cells were incubated with doxycycline for 5 h, rapamycin was added to one half of the culture, and the total rRNA content was assayed as described in the legend to Fig. 1. (C) Relative ratios of rRNA decay products to full-length rRNA in the cultures analyzed as described in the legend to panel B. Areas of gels indicated in panel A were used for the phosphorimager quantification of the decay products. Graphs show the averages for two cultures, normalized to the value at time zero with respect to rapamycin addition (5 h with doxycycline); error bars display ranges.

observed in rapamycin-treated cells, the *ski2Δ* and *ski7Δ* strains exhibited increased levels of 25S rRNA decay products (Fig. 6A, lanes 3 to 6). Accumulation was especially striking for the *25S(3') rRNA fragment, which became more abundant than the remaining intact 25S rRNA in *skiΔ* cells but did not accumulate in WT cells (Fig. 6A, compare lanes 1, 3, and 5).

We considered the possibility that the accumulation of rRNA decay intermediates might be due to ribosome surveillance by NRD (34), as saturated culture conditions could conceivably increase the number of nonfunctional ribosomes in cells. Although the nucleases that initiate NRD are unknown, the disassembly of ribosomal complexes stalled during translation requires Dom34 and Hbs1 (54), and the lack of Hbs1 was shown previously to increase the stability of 18S rRNA in NRD (17). We observed no significant changes in the extent or pattern of 18S or 25S rRNA decay in either *hbs1Δ* single mutants or *hbs1Δ ski2Δ* or *hbs1Δ ski7Δ* double mutants (Fig. 6A and B), suggesting that nutrient-controlled ribosome decay and NRD are triggered by distinct stimuli. Likewise, deletion of genes encoding Mms1 and Rtt101, two proteins implicated in 25S NRD (22), did not confer stabilization on rRNA after rapamycin treatment (Fig. 6B).

Because the data suggested that rapamycin-induced turnover is independent of systems that check ribosome functionality during translation, we asked whether translation itself is required for this process. Cycloheximide is a translation inhibitor that blocks elongation by eukaryotic ribosomes (50). To determine whether the lack of ongoing translation may affect ribosome stability in cells exposed to rapamycin, we added cycloheximide 15 min after rapamycin treatment and analyzed rRNA content 3 h later. While more than half of rRNA was degraded by this time in cells treated with rapamycin only, the addition of cycloheximide largely abolished the decrease in the rRNA content (Fig. 6C). In a control treatment with cycloheximide alone, rRNA was also stable during this time (Fig. 6C). New rRNA synthesis and maturation are known to cease rapidly in cycloheximide-treated cells because of lack of production of new proteins (55). Therefore, the stability of rRNA levels indicates that turnover of ribosomes is suppressed by cycloheximide treatment. Notably, cycloheximide was reported previously to stabilize mutant 18S rRNA but not mutant 25S rRNA in NRD (17). In rapamycin-treated cells, however, 25S and 18S rRNAs were stabilized to similar extents (Fig. 6C), again pointing to mechanistic differences between nutrient-dependent turnover and NRD. Unlike cycloheximide, both rapamycin and nutrient depletion have been shown to affect primarily initiation events in translation but neither blocks translation completely (5, 6, 10, 14, 16). Cycloheximide, in contrast, effectively stalls translating ribosomes on mRNAs (50). The stabilizing effect of cycloheximide on ribosomes thus suggests that rapamycin-induced rRNA decay depends on ongoing translation and that ribosomes locked onto mRNA during elongation may be resistant to degradation by cytoplasmic nucleases.

DISCUSSION

In yeast, activation of TOR in a nutrient-rich environment is responsible for directing a large part of cellular resources toward ribosome synthesis (38, 63, 65). The data obtained in this study suggest that inactivation of TOR not only stops the synthesis of new ribosomes, as previous studies have demonstrated (26, 45, 46), but also triggers extensive turnover of the existing ribosomes, resulting in a dramatic reduction of the cellular ribosome content.

FIG 5 Effects of the exosome cytoplasmic Ski adaptors on rRNA decay. (A) Accumulation of 25S rRNA decay products in *skiΔ* strains. RNA was analyzed as described in the legend to Fig. 4A; the WT strain is BY4741. (B) Time course of rapamycin treatment of *ski2Δ*, *ski7Δ*, and isogenic WT cells. RNA samples in the lanes are from equal volumes of culture. tRNA, which does not decrease in rapamycin-treated cells, was used to verify loading. (C) Relative ratios of 25S decay products to full-length 25S rRNA based on phosphorimager measurements of the gel areas indicated in panel B. (D) Quantitative analysis of full-length rRNAs and tRNA in rapamycin-treated strains represented in panel B. All graphs show the averages for two independent cultures relative to the average before treatment; error bars display ranges.

Our observations indicate that more than one half of all ribosomes present in a yeast culture grown in rich medium can be turned over within the first 5 to 6 h after exposure to rapamycin before the remaining ribosome pool becomes stabilized (Fig. 1). These data essentially imply that TOR-mediated control of the ribosome content is dual in nature: TOR signaling can affect both ribosome

synthesis and the degradation of mature ribosomes. From a theoretical perspective, such dual regulation may provide a more flexible strategy than the regulation of synthesis alone for fine tuning of the ribosome content in a rapidly changing environment. Previous studies of yeast indeed suggested a contributing role for ribosome degradation during transitions to a lower growth rate,

FIG 6 rRNA decay involves Ski2 and Ski7 during diauxic shift and is distinct from NRD. (A) rRNA decay products similar to those in rapamycin-treated cells accumulate after growth in YPD for 48 h in cells lacking *SKI2* or *SKI7* but are not significantly affected by deletion of *HBS1*. (B) rRNA remaining in strains lacking various 25S and 18S NRD genes after incubation with rapamycin for 5 h. The average for two independent cultures of each strain is shown; error bars indicate ranges. (C) Total rRNA content changes in cells treated with rapamycin, cycloheximide (Chx), or a combination of the two drugs. The average values for two cultures are shown; error bars indicate ranges.

which occur when the nutrient quality of growth medium begins to decline (28). Interestingly, ribosome degradation was also recently reported to take place in bacteria when culture conditions become limiting for exponential growth (43), suggesting an adaptive role for turnover as part of the dynamic control of the ribosome content across a broad range of microorganisms.

Given that more than 10^5 ribosomes are present in each cell of *S. cerevisiae* during growth in rich medium (62), the extent of the decrease in rRNA upon rapamycin treatment (Fig. 1D) implies turnover of tens of thousands of ribosomes per hour. Thus, yeast cells have a highly efficient system in place to degrade their ribosomes. In previous studies, ribosomes and ribosomal proteins were observed in the yeast vacuole when autophagy was activated by starvation conditions (32, 57). Data presented above, however, indicate that active autophagy is not a prerequisite for the rapid ribosome degradation in rapamycin-treated cells (Fig. 3). In addition, the finding that turnover of rRNA decay intermediates is influenced by the exosome together with its cytoplasmic cofactors (Fig. 4 and 5) argues that at least the initial steps of this process take place in the cytoplasm. These observations may not necessarily conflict with the findings in previous studies, as yeast cells may carry out ribosome degradation for different purposes by utilizing distinct mechanisms. For instance, targeting of ribosomes to the vacuole through autophagy may be important for recycling of the ribosomal material during long-term starvation or in preparation for sporulation as suggested previously (57), whereas cytoplasmic degradation may be used primarily for adjusting the size of the translation machinery in response to rapidly occurring changes in the nutrient environment.

One important question raised by our findings is what mechanism is responsible for switching from the stable state of ribosomes during steady growth to their degradation upon TOR inactivation. There is very little observable lag in the onset of rRNA degradation after rapamycin treatment (Fig. 1D), suggesting that the machinery for the rapid ribosome degradation may already exist in growing cells even though during exponential growth ribosomes appear to undergo little turnover (Fig. 1E). Inhibition of translation initiation is a well-documented effect of rapamycin (6) and is also characteristic of conditions of nutrient downshifts (5, 10, 14). Inhibition of initiation would be expected to create a pool of ribosomal subunits that have finished previous rounds of translation but cannot start another translation job. Hence, one attractive hypothesis is that yeast cells may have a system that recognizes such idle subunits and targets them for degradation. Stabilization of rRNA in rapamycin-treated cells after treatment with cycloheximide (Fig. 6C) lends support to this idea, as cycloheximide prevents the runoff of translating ribosomes from mRNA, and this might protect them from degradation by keeping subunits sequestered away from the idle pool. Future studies will be needed to test this hypothesis.

The evidence of the cytoplasmic rRNA turnover in a eukaryotic organism suggests intriguing parallels in mechanisms of nutrient-dependent ribosome degradation between eukaryotes and prokaryotes, as the latter use a variety of nucleases in this process (18, 69). Our data show that the exosome, Ski7, and apparently the entire SKI complex of exosome cofactors function by promoting rRNA decay (Fig. 4 and 5). Interestingly, the exosome core contains subunits homologous to the prokaryotic RNase PH (48), which was recently shown to play an important role in rRNA degradation in *Escherichia coli* (8). The Ski-exosome system in

yeast appears to function largely at later stages of 25S rRNA degradation than RNase PH, suggesting that additional nucleases may work upstream to initiate this process in eukaryotes. Our analysis of other exonucleases involved in rRNA decay showed that the 5' exonuclease Xrn1 can also play a role in the degradation of rRNA, although its activity appears to be highly dependent on the strain background (D. G. Pestov and N. Shcherbik, unpublished observations), suggesting that pathways to degrade rRNA fragments in the cytoplasm may be flexible and utilize multiple nucleases.

Defective cytoplasmic ribosomes containing mutations in rRNA were recently found to be selectively degraded through a process termed nonfunctional rRNA decay (17, 22, 34). Our analysis of strains deficient in known genes involved in NRD (Fig. 6A and B) indicates that nutrient-dependent turnover is controlled separately, although we cannot exclude the possibility that the two processes may intersect at some point. Both NRD and nutrient-dependent turnover are likely to involve additional, early-acting nucleases that remain to be identified. Endonucleases represent one possible group of candidate factors that could initiate RNA decay; however, such nucleases have to date remained elusive. Another possibility is that proteins that do not possess nucleolytic activity themselves, such as helicases, could initiate ribosome turnover by stripping components that protect rRNA in mature ribosomes, thereby making rRNA susceptible to attack by nonspecifically acting nucleases. The mutants unable to efficiently process rRNA decay intermediates, such as *skiΔ* strains deficient in 25S rRNA decay (Fig. 5A and 6A), should facilitate future searches for factors that act at early steps of eukaryotic ribosome degradation. Because ribosomes are ribonucleoprotein particles, we also anticipate that additional systems exist in cells to process ribosomal proteins removed from disassembled cytoplasmic ribosomes, and the factors involved in such activities also await identification and analysis.

Finally, it will be interesting to extend analysis of rRNA degradation to metazoan cells. Increased rRNA turnover was observed previously in contact-inhibited mammalian cells (64) and cells undergoing differentiation (11). In addition, recent studies of human cells revealed the presence of diverse rRNA fragments in the cytoplasm and the increase of such fragments after knockdowns of scavenging nucleases (56). Thus, it is possible that a process analogous to the cytoplasmic ribosome turnover described herein contributes to the control of the ribosome content in mammalian cells.

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