The Saccharomyces cerevisiae 60 S Ribosome Biogenesis Factor Tif6p Is Regulated by Hrr25p-mediated Phosphorylation*

Received for publication, December 18, 2007, and in revised form, January 24, 2008 Published, JBC Papers in Press, February 5, 2008, DOI 10.1074/jbc.M710294200

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The biosynthesis of 60 S ribosomal subunits in Saccharomyces cerevisiae requires Tif6p, the yeast homologue of mammalian eIF6. This protein is necessary for the formation of 60 S ribosomal subunits because it is essential for the processing of 35 S pre-rRNA to the mature 25 S and 5.8 S rRNAs. In the present work, using molecular genetic and biochemical analyses, we show that Hrr25p, an isoform of yeast casein kinase I, phosphorylates Tif6p both in vitro and in vivo. Tryptic phosphopeptide mapping of in vitro phosphorylated Tif6p by Hrr25p and 32P-labeled Tif6p isolated from yeast cells followed by mass spectrometric analysis revealed that phosphorylation occurred on a single tryptic peptide at Ser-174. Sucrose gradient fractionation and coimmunoprecipitation experiments demonstrate that a small but significant fraction of Hrr25p is bound to 66 S preribosomal particles that also contain bound Tif6p. Depletion of Hrr25p from a conditional yeast mutant that fails to phosphorylate Tif6p was unable to process pre-rRNAs efficiently, resulting in significant reduction in the formation of 25 S rRNA. These results along with our previous observations that phosphorylatable Ser-174 is required for yeast cell growth and viability, suggest that Hrr25p-mediated phosphorylation of Tif6p plays a critical role in the biogenesis of 60 S ribosomal subunits in yeast cells.

Eukaryotic translation initiation factor 6 (eIF6)3 was initially purified as a protein that can bind the 60 S ribosomal subunit and prevent its association with the 40 S ribosomal subunit (1–4). Based on this ribosomal subunit anti-association property, the protein was originally thought to be an initiation factor that functions to provide a pool of free ribosomal subunits required for initiation of protein synthesis (5). The protein was named eIF6, although a role in translation was not demonstrated in these earlier studies. To understand the function of this protein in translation, Si et al. (6) first cloned the human cDNA and then the yeast Saccharomyces cerevisiae gene (7) encoding functionally active eIF6, each of 245 amino acids. The two proteins are 72% identical. The yeast gene, designated TIF6, is a single copy gene that is essential for cell growth and viability (7). These properties of TIF6 allowed the construction of a conditional null allele by placing its expression under the control of the regulatable GAL10 promoter. Depletion of TIF6 in this yeast mutant strain inhibited the rate of in vivo protein synthesis (7). However, a more detailed analysis of the protein synthesis parameters in TIF6-depleted cells showed that the reduced rate of protein synthesis was not due to a direct inhibition in initiation (7). Rather, the biogenesis of 60 S ribosomal subunits was severely inhibited. Similar observations were also reported by Sanvitto et al. (8), who identified eIF6 from mammalian cells as a β4 integrin-interacting protein. Specifically, lack of TIF6 in yeast cells prevented the processing of pre-ribosomal RNA (pre-rRNA) to the mature 25 S and 5.8 S rRNAs, the constituents of the 60 S ribosomal particle (9). In agreement with these observations, TIF6 was found to be a constituent of a multiprotein assembly complex associated with 60 S pre-ribosomal particles (also known as the 66 S pre-ribosomal particles) in the nucleolus that are the intermediates in the biosynthesis of mature 60 S ribosomal subunits (7, 10).

In previous studies we have observed that in both mammalian and yeast cells, eIF6 (TIF6) is phosphorylated, and we have identified casein kinase 1 α (CK 1α) as the protein kinase responsible for this modification in mammalian cell extracts (11). The sites of in vitro phosphorylation in mammalian eIF6 were identified as the serine residues at positions 174 (major site accounting for >90% of the total in vitro phosphorylation) and 175 (<10% phosphorylation). The Ser residue at position 174 is present in a highly conserved consensus CK I sequence from yeast to mammals (11). Mutation of Ser-174 to alanine abolished phosphorylation of TIF6 in yeast cells by 75% and caused a loss of cell growth and viability (11). When both Ser-174 and Ser-175 were mutated to alanine, phosphorylation was virtually abolished (11). These observations suggested that phosphorylatable serine residues at Ser-174 and Ser-175 play an important regulatory role in the function of TIF6 in yeast cells. However, the protein kinase(s) responsible for phosphorylation of TIF6 in yeast has not been identified.

In the present work we have carried out molecular genetic and biochemical analyses to show that Hrr25p, an isoform of the budding yeast CK I (12) that is encoded by an essential gene

* This research was supported by National Institutes of Health Grant GM15399 and by NCI, National Institutes of Health Cancer Core Support Grant P30CA13330. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: eIF, eukaryotic (translation) initiation factor; CK I, casein kinase I; ORF, open reading frame; Ni-NTA, nickel-nitrilotriacetic acid; HA, hemagglutinin; DTT, dithiothreitol; YPD, yeast extract/peptone/dextrose; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TAP, tandem affinity purification; GFP, green fluorescent protein; premRNA, pre-ribosomal RNA; TCPK, l-1-tosylamido-2-phenylethyl chloromethyl ketone.

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Hrr25, phosphorylates Tif6p at Ser-174 in vitro and in vivo. Tryptic phosphopeptide mapping shows that this is the only Tif6p site phosphorylated in yeast cells. We also show that a small but significant fraction of Hrr25p localizes along with Tif6p on 6S pre-ribosomal particles. Furthermore, Hrr25p-mediated phosphorylation is also required for efficient processing of pre-rRNAs to form the mature rRNAs. These results suggest that constitutively active kinase Hrr25p plays an important regulatory role in coordinating the process of 60S and 40S ribosome biogenesis in yeast.

**EXPERIMENTAL PROCEDURES**

**Media, Growth Conditions, and Genetic Methods—**Yeast strains were grown at 30°C in standard media as described previously (7). For pre-rRNA processing experiments using [methyl-3H]methionine pulse, synthetic complete medium lacking methionine and containing either 2% galactose (SGal) or 2% dextrose (SD) was used as the carbon source. These methionine-lacking media were designated SGal-Met and SD-Met, respectively. Yeast genetic methods as well as methods for isolation of plasmids and genomic DNAs, cloning, and bacterial transformation were carried out as described (13, 14).

**Yeast Strains—**The genotype of yeast strains used in this work are described in Table 1. The construction of yeast strains used in this study is as follows. For epitope-tagging of chromosomal genes at the 3′ ends of their open reading frames (ORFs), the PCR-based gene targeting method of Longtine et al. (15) was used. The construction of several other strains were as follows. For overexpression of hemagglutinin (HA)-tagged Hrr25p in yeast cells, the ORF of HRR25 was amplified by PCR from yeast genomic DNA using *Pyrococcus* DNA polymerase (Stratagene) using two primer sequences as follows; N terminus 5′-dgcggatcatgtgactaaagtag-3′ having a BamHI overhang and C terminus 5′-dtgacgagcttacaacaattgactggca-3′ having a NotI overhang. The PCR product was used in a three fragment ligation reaction with a NotI/SacI fragment derived from the vector pGEXYP1 and URA3-based CEN plasmid pRS316GAL digested at the BamHI and SacI sites, respectively. Yeast genetic methods as well as methods for isolation of plasmids and genomic DNAs, cloning, and bacterial transformation were carried out as described (13, 14).

**Isolation of Hrr25p and Yck1p**

**Preparation of Hrr25p-depleted Yeast Cell-free Extracts—**Exponentially growing cells (A_600_ = 0.2) of wild-type YPH499 (+ Hrr25p) and the isogenic conditional mutant strain KKY387 in YPGal media were each filtered through a sterile 0.2-μm filter and then suspended in YPD media and allowed to grow for about 8 h (A_600_ of about 0.6) until there was no detectable Hrr25p in the mutant strain as determined by Western blot analysis of cell lysates. Harvested cells were lysed in buffer containing 20 mM Tris- HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 10 mM MgCl_2_, and phosphatase inhibitors (50 mM NaF, 50 mM sodium molybdate) and used as a source of protein kinase for Tif6p phosphorylation. It should be noted that the growth of KKY387 cells begins to slow down 12–14 h after transfer to SD medium.

**Coimmunoprecipitation of Tif6p with Hrr25p—**A 100-ml culture of exponentially growing PRY101 yeast cells was harvested, washed twice with ice-cold water, and then suspended in 500 μl of a coimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 10 mM MgCl_2_, and an aliquot of a mixture of protease inhibitors). Cells were disrupted by vortexing with glass beads and centrifuged. The glass beads were washed once with 500 μl of the coimmunoprecipitation buffer, and the supernatant obtained after centrifugation was mixed with the initial supernatant. The combined supernatant (about 1 ml) was incubated with 10 μl of agarose-
conjugated anti-HA antibody (Santa Cruz) for about 4 h with gentle mixing at 4 °C. Immunocomplexes bound to agarose beads were isolated by centrifugation and washed with 0.5 ml of the communoprecipitation buffer (twice). Proteins bound to the immunocomplexes were eluted by suspending the beads in 30 µl of 125 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol and incubating the suspension in a boiling water bath for 5 min. After centrifugation, the supernatant was treated with 100 mM DTT, cooled, and then subjected to SDS-PAGE (15% gel). The resolved proteins were transferred electrophoretically to a polyvinylidene difluoride membrane. The washed membrane was then analyzed by Western blotting using appropriate antibodies.

**Assay for in Vitro Phosphorylation of Tif6p**—Phosphorylation of Tif6p was carried out in reaction mixtures (30–50 µl each) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM DTT, 10 mM MgCl$_2$, 100 µM [γ-32P]ATP (10,000 cpm/pmol), 15 pmol of recombinant His$_6$-Tif6p, a mixture of phosphatase inhibitors (50 mM NaF, 50 mM sodium molybdate), and the indicated amounts of either yeast cell-free extracts or the purified kinase protein. In some experiments the nuclear or cytoplasmic fractions of yeast cell-free extracts replaced the total yeast cell-free extracts as the source of kinase. After incubation at 30 °C for 30 min, each reaction was treated with 450 µl of buffer I (20 mM potassium phosphate, pH 7.8, 0.5 M NaCl, 6 mM imidazole-HCl, pH 8.0, and 10% glycerol) followed by the addition of 15 µl of equilibrated Ni-NTA-agarose bead suspension. The solutions were mixed gently at 4 °C for 2 h then centrifuged. The agarose beads were washed twice with 1 ml of buffer I containing 50 mM imidazole. The washed beads containing bound His$_6$-Tif6p were suspended in 50 µl of SDS-loading buffer, boiled, and then subjected to SDS-PAGE (either 15% gel or 4–15% gradient gel as indicated). For reactions using the purified kinase, the incubated reaction mixtures were directly treated with SDS-loading buffer, boiled, and then subjected to SDS-PAGE. In each case, after SDS-PAGE, the resolved proteins were transferred to a polyvinylidene difluoride membrane. The membrane was analyzed by autoradiography to determine the extent of phosphorylation of Tif6p. Subsequently, the recovery of Tif6p in each reaction was determined by Western blot analysis using anti-His antibody.

**Phosphopeptide Mapping**—The procedure for the preparation of tryptic phosphopeptides from immunocomplexes of in vivo 32P-labeled Tif6p was similar to that described previously (18). For two-dimensional separation of tryptic phosphopeptides by thin-layer chromatography, the samples were spotted 4 cm from the bottom edge of a 20 × 20-cm thin layer cellulose plate (Fisher) and electrophoresed at pH 1.9 in 2.5% formic acid, 17.8% acetic acid for 20 min at 16 °C at 1000 V. The plates were air-dried and then subjected to ascending chromatography in pyridine:acetic acid-1-butanol:H$_2$O (50:15:75:60). The air-dried plates were subsequently subjected to autoradiography using phosphorimaging.

**Tandem Affinity Purification and Identification of Tif6p-associated RNA and Protein Components**—The genomic copy of TIF6 of the yeast strain W303α (Table 1) was C-terminal-tagged with the TAP-tag cassette using PCR-based gene targeting methods (15). The resulting yeast strain, designated PRY104 (Table 1), expresses TAP-tagged Tif6p from its genomic promoter. This strain grew at a rate similar to the untagged wild-type strain. Immunoblot analysis of extracts of this strain showed a protein band whose size corresponded to Tif6p-TAP fusion protein. Furthermore, the Tif6p-TAP fusion protein sedimented in sucrose gradients primarily at the 60 S–66 S region (data not shown). Cells expressing Tif6p-TAP were grown in YPD at 30 °C to an A$_{600}$ of about 1.0, and cell-free extracts were subjected to tandem affinity purification as described by Rigaut et al. (19). RNAs that co-purified with the Tif6p-TAP complex(s) were extracted from the final eluate with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and analyzed by Northern blot by using 32P-labeled deoxyoligonucleotide probes (9) that specifically detect each pre-rRNA species. Primer extension was carried out as described by Venema et al. (20). The probe used for detection of 35 S and 25.5 S pre-rRNAs by primer extension was (5’-ACCGCTGTATAGACTAGGC-3’) and (5’-CGCTTAGACGCTCTCTTTC-3’), respectively, as described by these investigators (20).

**Other Materials and Methods**—Recombinant yeast His$_6$-Tif6p was purified from BL21 (DE3) cells carrying the ORF of yeast Tif6p in the plasmid pRSET-A by following a procedure similar to that described previously for the purification of His$_6$-tagged mammalian elf6 (11). The procedure involved affinity purification from a Ni-NTA column followed by gradient elution from an fast protein liquid chromatography-Mono Q column. The final preparation was >95% pure as judged by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Nuclear and cytoplasmic extracts from exponentially growing yeast cells were prepared by an adaptation of the method of Evans and Engelke (21). The procedures used for pulse-chase labeling of pre-rRNAs with [methyl-3H]methionine and subsequent analysis of RNA by gel electrophoresis were as described (9). RNA was isolated from each immunocomplex by a phenol/chloroform extraction procedure as described (9). Purified yeast elf5 (Tif5p) was a kind gift of Drs. Michael Acker and Jon Lorsch of the Johns Hopkins University of Medicine, Baltimore, MD. Anti-Pgk1p antibodies were purchased from Molecular Probes, whereas anti-Nop1p antibodies were obtained from EnCor Biotechnology Inc. Anti-Tcm1p (L3) antibodies and Northern probes JW71 and JW499 were kind gifts of Dr. Jonathan Warner of this institution.

**RESULTS**

**Hrr25p Phosphorylates Tif6p in Vitro**—To identify the kinase responsible for phosphorylation of Tif6p in vitro and to characterize the phosphorylation reaction, yeast cell-free extracts were fractionated into nuclear and cytosolic fractions. The validity of the fractionation was verified by using antibodies for 3-phosphoglycerate kinase, Pgk1p (a marker for cytoplasmic proteins), and Nop1p (a marker for nuclear proteins) (Fig. 1, panel C). Using bacterially expressed recombinant His$_6$-Tif6p as the substrate and [γ-32P]ATP as the phosphoryl donor and increasing concentrations of either the nuclear or cytoplasmic extract as the source of the kinase, we observed that Tif6p was readily phosphorylated by the nuclear extract (Fig. 1, panel A,
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FIGURE 1. Tif6p is phosphorylated by a nuclear kinase in yeast cells. A, the preparation of reaction mixtures (50 μl each) containing either yeast nuclear or cytoplasmic extracts and the subsequent analysis of phosphorylation of Tif6p were as described under “Assay for In Vitro Phosphorylation of Tif6p” under “Experimental Procedures.” Where indicated, 15 pmol of recombinant His6-Tif6p were added to the reactions. The amount of nuclear and cytoplasmic extracts added were as follows: lane a, 1 μg; lane b, 1 μg; lane c, 2 μg; lane d, 4 μg; lane e, 8 μg; lane f, 20 μg; lane g, 10 μg; lane h, 20 μg; lane i, 50 μg. B, the recovery of His6-Tif6p in each reaction was determined by Western blot analysis using anti-His antibodies. C, nuclear (lane a) and cytoplasmic extracts (lane b) (30 μg each) were subjected to Western blot analysis using anti-Nop1p and anti-Pgk1p antibodies, respectively.

lanes d and e). In contrast, under the same conditions the relative efficiency of phosphorylation of Tif6p by the cytosolic fraction was low (Fig. 1A, lane h). A higher concentration of the cytosolic fraction was inhibitory in the phosphorylation reaction (Fig. 1A, lane i). The presence or absence of phosphatase inhibitors did not have any effect on the extent of phosphorylation (data not shown). Furthermore, the amount of His6-Tif6p recovered from each reaction was similar (Fig. 1, panel B).

It has been reported previously that mammalian eIF6 is phosphorylated at Ser-174 by CK Iα (11). In view of our observation (11) that this serine residue is present in a highly conserved consensus CK I sequence (12) between yeast and mammals, we initially investigated whether a particular isoform(s) of yeast CK I can phosphorylate Tif6p in vitro. In S. cerevisiae there are four different isoforms of CK I, each encoded by a separate gene, YCK1, YCK2, YCK3, and HRR25 (12, 22–24). Among these isoforms, Hrr25p localizes throughout the cell (16), whereas Yck1p, Yck2p, and Yck3p are prenylated and tightly bound to the plasma membrane (24, 25). To show if any isoform of yeast CK I is able to phosphorylate Tif6p in vitro, we purified Hrr25p, Yck1p, and Yck2p and used these purified proteins to phosphorylate recombinant Tif6p (Fig. 2). Tif6p was readily phosphorylated by both immunopurified Hrr25p (Fig. 2A) and by bacterially expressed recombinant GST-Hrr25p (Fig. 2B). In contrast, the other purified CK I isoform, Yck1p, was unable to phosphorylate Tif6p (Fig. 2C). Similar observations were made using β-galactosidase fusion protein of Yck2p (22, 23) as the source of the kinase (data not shown). Both Yck1p (Fig. 2D, lanes b and c) and Yck2p (data not shown) were, however, able to phosphorylate cascin, a model in vitro substrate, for all isoforms of CK I, indicating that these kinases were enzymatically active. It should be noted that with all the isoforms of CK I tested, an additional higher molecular weight band (apparent Mr of about 55,000–58,000) was observed in all reactions (Fig. 2). Presumably, this band represents the autophosphorylated form in each case. All the isoforms of yeast CK I including Hrr25p are known to auto-phosphorylate and migrate in SDS-PAGE with an apparent Mr of about 55,000–58,000 (12, 26).

Tif6p Is Phosphorylated by Hrr25p in Yeast Cell Lysates—Additional confirmation that Hrr25p is indeed involved in phosphorylation of Tif6p in yeast cells came from comparison of the ability of the cell-free extracts isolated from two isogenic yeast strains YPH499 and KKY387 that differ in their conditional expression of Hrr25p (Table 1). In the strain KKY387, the genomic copy of HRR25 was deleted, and the essential function of HRR25 provided from a 2-μm plasmid in which Hrr25p was expressed from a galactose-inducible GAL10 promoter as an N-terminal-ubiquitinylated HA-tagged fusion protein (16). The presence of this N-terminal module (degron) results in rapid degradation of Hrr25p when the strain is grown in YPD (glucose) medium. In contrast, in the isogenic wild-type strain YPH499, Hrr25p is expressed from its genomic promoter as an untagged protein.

To investigate the role of Hrr25p in the phosphorylation of Tif6p, exponentially growing cultures of YPH499 and KKY387 growing in YPGal(galactose) medium were each divided into two parts. One part of each culture was maintained in YPGal medium, whereas the other part was shifted to a YPD medium and allowed to grow for 8 h to deplete Hrr25p from KKY387 cells (Fig. 3, panel B). Cell-free extracts prepared from each condition were then used as the source of protein kinase for in vitro phosphorylation of Tif6p (Fig. 3). Depletion of Hrr25p in the conditional mutant strain KKY387 resulted in severe inhibition of 32P incorporation into Tif6p (Fig. 3A, compare lanes b and c). Longer exposures failed to show any band corresponding to Tif6p in the Hrr25p-depleted cell extract lane. In contrast, extracts prepared from the isogenic wild-type strain YPH499, which contains the wild-type copy of HRR25, were active in phosphorylation of Tif6p (Fig. 3A, lane a) when subjected to the same YPGal to YPD media shift. It should be noted that this effect on Tif6p phosphorylation by Hrr25p is specific since phosphorylation of either yeast eIF5 (Tif5p) that is phosphorylated by casein kinase II (18) or the model substrate casein, which is known to be phosphorylated by all the isoforms of CK I as well as by CK II, is not significantly affected by the depletion of Hrr25p from yeast cells (Fig. 3, C and D, respectively). These results show that Hrr25p phosphorylates Tif6p in yeast cell lysates.

Hrr25p Phosphorylates Tif6p in Vivo and in Vitro at the Same Site—The sites in Tif6p phosphorylated in vivo and in vitro were determined by two-dimensional phosphopeptide mapping. 32P-Labeled Tif6p isolated from KSY607 yeast cells (Table 1) was digested with excess TPCK-trypsin, and the resulting
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results suggest that Hrr25p phosphorylates Tif6p in vivo in yeast cells and that phosphorylation occurs at one or more serine residues present in the same tryptic peptide.

To identify the peptide(s) containing the phosphorylated serine residues, recombinant Tif6p was maximally phosphorylated in vitro with the purified kinase, and the \(^{32}P\)-labeled Tif6p was isolated by SDS-PAGE. Gel slices containing either the phosphorylated or unphosphorylated Tif6p (50 pmol each) were reduced, alkylated, and digested with Glu-C protease, which cleaves at the C termini of glutamic acid residues of the protein. Each digestion product was analyzed by MALDI-TOF with a Perseptive MALDI-TOF DE-STR mass spectrometer. From Glu-C digestion products of unphosphorylated Tif6p, two ions were observed at 4173.8 and 4189.8 Da corresponding to masses of the peptide Leu-173–Glu-214 and its methionine residue-oxidized form, respectively. In contrast, from the digestion products of phosphorylated Tif6p, two new ions at 4524.2 and 4270.2 Da corresponding to the addition of a phosphate onto the peptide Leu-173–Glu-214 and its methionine residue-oxidized form, respectively, were observed. These two ions were absent in the products obtained from the digests of the unphosphorylated Tif6p (Fig. 4D, compare the upper and lower panels).

To determine the phosphorylation site(s), the products of Glu-C digestion were further cleaved by trypsin, resulting in the formation of two peptide ions 1666.99 and 1747.01 corresponding to the peptide Leu-173–Arg-188 and its phosphorylated form. This result demonstrates that the phosphorylation site is either Ser-174 or on Ser-175. Using a combination of chemical derivation that converts the phosphoserine-containing peptides to propanoylcysteine-containing peptides and tandem mass spectrometry, we identified that Ser-174 was the major phosphorylation site (data not shown).

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phosphopeptides were separated by a combination of high voltage electrophoresis and thin layer chromatography. One major phosphopeptide was detected (Fig. 4A, panel b), suggesting that phosphorylation of Tif6p occurred primarily within one tryptic peptide. In a separate experiment, phosphoamino acid analysis of the same sample of Tif6p showed that serine was the only amino acid residue phosphorylated (data not shown). When recombinant \(\text{His}_{6}\)-Tif6p was phosphorylated in vitro by immunopurified HA-tagged Hrr25p and the resulting \(^{32}P\)-labeled Tif6p was subjected to two-dimensional tryptic peptide mapping, the same distinct phosphopeptide observed in vivo was resolved (Fig. 4A, panel a). When the tryptic peptides obtained from \(^{32}P\)-labeled Tif6p isolated from yeast cells were mixed with those obtained from Tif6p labeled in vitro with immunopurified HA-Hrr25p, an identical phosphopeptide was resolved (Fig. 4A, panel c). These phosphopeptide mapping, the same distinct phosphopeptide was detected (Fig. 4A, panel c), suggesting that Hrr25p phosphorylates Tif6p in vivo in yeast cells and that phosphorylation occurs at one or more serine residues present in the same tryptic peptide.

To identify the peptide(s) containing the phosphorylated serine residues, recombinant Tif6p was maximally phosphorylated in vitro with the purified kinase, and the \(^{32}P\)-labeled Tif6p was isolated by SDS-PAGE. Gel slices containing either the phosphorylated or unphosphorylated Tif6p (50 pmol each) were reduced, alkylated, and digested with Glu-C protease, which cleaves at the C termini of glutamic acid residues of the protein. Each digestion product was analyzed by MALDI-TOF with a Perseptive MALDI-TOF DE-STR mass spectrometer. From Glu-C digestion products of unphosphorylated Tif6p, two ions were observed at 4173.8 and 4189.8 Da corresponding to masses of the peptide Leu-173–Glu-214 and its methionine residue-oxidized-form, respectively. In contrast, from the digestion products of phosphorylated Tif6p, two new ions at 4524.2 and 4270.2 Da corresponding to the addition of a phosphate onto the peptide Leu-173–Glu-214 and its methionine residue-oxidized form, respectively, were observed. These two ions were absent in the products obtained from the digests of the unphosphorylated Tif6p (Fig. 4D, compare the upper and lower panels).

To determine the phosphorylation site(s), the products of Glu-C digestion were further cleaved by trypsin, resulting in the formation of two peptide ions 1666.99 and 1747.01 corresponding to the peptide Leu-173–Arg-188 and its phosphorylated form. This result demonstrates that the phosphorylation site is either Ser-174 or on Ser-175. Using a combination of chemical derivation that converts the phosphoserine-containing peptides to propanoylcysteine-containing peptides and tandem mass spectrometry, we identified that Ser-174 was the major phosphorylation site (data not shown).

These results are in agreement with our previous observation (11) that mutation of Ser-174 to alanine leads to >75% abolition of phosphorylation of Tif6p in vivo in yeast cells and caused loss of cell growth and viability. In contrast, mutation of Ser-175 alone inhibited phosphorylation only marginally (about 10–15% inhibition) and did not cause loss of viability of yeast
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**TABLE 1**

| Yeast strains used | Genotype | Reference or source |
|--------------------|----------|---------------------|
| W303 α             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 | 13 |
| KSY603             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLIRA GAL10::3HA-HRR25) | 7 |
| KSY606             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pTRPI TIF6-myc) | 11 |
| KSY607             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3, p[LEU2::TIF6-HA] | 11 |
| UBY166             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pTRPI TIF6-myc) (pLIRA GAL10::HRR25-HA) | This work |
| YPH999             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | 16 |
| KKY387             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY101             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY102             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY103             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY104             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY105             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |
| PRY106             | MATα leu2-3,112 his3-1, 15 ade2-1 trp-1 ura3-1 can1-100 tif6::HIS3 (pLEU2 GAL10::3HA-HRR25) | This work |

To determine the ribosomal particles on which Tif6p is associated, we used TAP protocol (19) to purify proteins and pre-rRNA(s) associated with the Tif6p-TAP purified complex from lysates of PRY104 yeast cells expressing Tif6p-TAP. The RNA that copurified with the Tif6p-TAP complex was subjected to Northern and primer extension analysis followed by phosphorimaging quantification. The RNA isolated from whole-cell extracts and untagged strains was also analyzed as controls. The results presented in Fig. 6, A and B, show that there was a selective enrichment of 27 S, 25.5 S, and 7 S pre-rRNAs in the Tif6p-TAP-purified complex. However, no 35 S pre-ribosomal RNA was detected in the purified complex (Fig. 6, panel B). Based on the relative enrichment of 27 S, 25.5 S and 7 S pre-rRNAs, we suggest that Tif6p is predominantly associated with two late 66 S pre-ribosomal particles, one containing 27 SB pre-rRNA and the other with more mature pre-60 S ribosomal particles containing 25.5 S and 7 S pre-rRNA(s). However, the absence of 35 S pre-rRNA(s) in the Tif6p-TAP associated complex suggests that Tif6p is not present in the 90 S particle (Fig. 6B).
Phosphorylation of Yeast Tif6p by Hrr25p

To determine whether Hrr25p could be associated with the 66 S pre-ribosomal particles, we investigated whether Tif6p and Hrr25p can be coimmunoprecipitated from cell extracts. For this purpose, extracts of PRY101 expressing HA-tagged Tif6p and Myc-tagged Hrr25p were immunoprecipitated with anti-HA antibody. Analysis of the immunocomplex by SDS-PAGE followed by immunoblotting with anti-HA and anti-Myc antibodies demonstrated that both Tif6p and Hrr25p were present in the immunocomplex (Fig. 6C, lane f). Similar results were obtained when the coimmunoprecipitation reaction was carried out with anti-Myc antibodies (data not shown). When the RNA samples isolated from the immunocomplex, obtained using either anti-HA or anti-Myc antibodies, were analyzed by Northern blot hybridization using appropriate DNA probes, the presence of 27 S pre-rRNA was readily detected (Fig. 6D, lanes d and b, respectively). These results indicate that Hrr25p that was coimmunoprecipitated with Tif6p was associated with the 66 S pre-ribosomal particles. In contrast, whereas 20 S pre-rRNA was detected when Hrr25p was immunoprecipitated with anti-Myc antibodies, no 20 S pre-rRNA was detected in the immunocomplex precipitated with HA-Tif6p (Fig. 6D, lanes b and d), indicating that unlike Hrr25p, Tif6p does not associate with the pre-40 S ribosomal particles. It should be noted that the amount of 27 S pre-rRNA immunoprecipitated with HA-tagged Tif6p was significantly higher than that immunoprecipitated with Myc-tagged Hrr25p. These results suggest that although a majority of cellular Tif6p is associated with 66 S preribosomal particles, only a small fraction of Hrr25p associates with these 66 S particles at any one time.

Hrr25p Is Required for Pre-rRNA Processing and Consequently for Ribosome Biogenesis—An important function of Tif6p in yeast cells is its association with the 66 S preribosomal particles (7, 10) and its participation in the processing of 27 S pre-rRNA to mature 25 S and 5.8 S rRNAs (9). In view of our observations presented here that Hrr25p is the kinase responsible for phosphorylating Tif6p in yeast cells, we investigated whether depletion Hrr25p, which should give rise to unphosphorylated Tif6p in yeast cells, inhibited the processing of 27 S pre-rRNA. For this purpose exponentially growing cultures of the yeast strain PRY103 ( [ hrr25::lox-P-KanMX-loxP p [ LEU2 GAL10::3HA-HRR25 degron TIF6-myc13::HIS3 ] ] in SGal-Met medium containing a conditional Hrr25p expression system and the corresponding isogenic wild-type strain PRY102 ([ HRR25 TIF6-myc13::HIS3 ] ) in SGal-Met media were each transferred to SD-Met (glucose) medium, and the cells were allowed to grow for 8 h to deplete Hrr25p from the PRY103 cells. Cells were then pulse-labeled with [ 3H-methyl ] methionine for 2.5 min and chased for 3 and 10 min with an excess of unlabeled methionine. [Because 25 S and 18 S rRNAs are highly
and specifically methylated, the processing of pre-rRNAs can be readily monitored by labeling with [methyl-\(^{3}H\)]methionine (29). Analysis of the total \(^{3}H\)-RNA samples isolated from each batch of cells by formaldehyde agarose gel electrophoresis followed by fluorography showed that in the wild-type PRY102 cells, the 35 S pre-rRNA and the 27 S and 20 S processing intermediates were rapidly chased into mature 25 S and 18 S rRNAs, as expected (Fig. 7, Panel A). In contrast, in Hrr25p-depleted mutant PRY103 cells, the rates of formation of all pre-rRNA intermediates were slower. There was a significant decrease in the conversion of 35 S pre-rRNA to 27 S and 20 S pre-rRNAs and the formation of 18 S mature rRNA was severely inhibited. This observation is in agreement with that of Schafer et al. (28) who reported that Hrr25p-mediated phosphorylation of several pre-40 S ribosomal proteins is essential for the formation of 18 S rRNA and consequently for 40 S ribosome biosynthesis. More importantly, in these Hrr25p-depleted cells, the formation of mature 25 S rRNA was also inhibited, albeit to a lesser extent than that of 18 S rRNAs (Fig. 7, Panel A).

One striking aspect is that in Hrr25p-depleted cells, the processing of 35 S pre-rRNA leads to significant loss of the RNA rather than the production of 27 S, 18 S and 25 S mature RNA species suggesting degradation of the unprocessed pre-RNAs that presumably accumulates in the absence of Hrr25p. Similar degradation of unprocessed pre-rRNAs were observed previously (9).
Phosphorylation of Yeast Tif6p by Hrr25p

To show that the reduction in the formation of 25 S rRNA was due to lack of Hrr25p-mediated Tif6p phosphorylation, we carried out [methyl-3H]methionine pulse-chase experiments in yeast cells under conditions where only phosphorylation of Tif6p by Hrr25p is blocked without affecting phosphorylation of any other cellular substrates. For this purpose, we constructed a yeast strain (tif6::HIS3 p[URA3 GAL10::Ub-HA-TIF6] p[TRP1 tif6-GFP]) expressing both the wild-type and the conditional mutant alleles of tif6p (SI74A, S175A) from two separate CEN plasmids. The resulting strain PRY106 expressed the wild-type Tif6p from a GAL10 promoter as an N-terminal-ubiquitinylated HA-tagged fusion protein and the mutant tif6p (S174A, S175A) as a C-terminal GFP-tagged fusion protein from its endogenous promoter. As a control, we constructed another yeast strain PRY105 (tif6::HIS3 p[URA3 GAL10::Ub-HA-TIF6] p[TRP1 TIF6-GFP]) which is isogenic to the strain PRY106 except that it expresses wild-type Tif6p from its endogenous promoter instead of the phosphomutant form of Tif6p.

Exponentially growing cultures of both PRY105 and PRY106 strains in SGal-Met media were each transferred to SD-Met (glucose) media, and the cells were allowed to grow for 2 h to deplete HA-tagged Tif6p from both the PRY105 and PRY106 cells (Fig. 7C). Under these conditions, the expression of GFP-tagged Tif6p phosphomutant in the PRY106 cells as well as that of GFP-tagged wild-type Tif6p in PRY105 cells remained uninhibited (Fig. 7C). Each batch of cells was then pulse-labeled with [methyl-3H]methionine for 2.5 min and then chased for 0, 3, and 10 min with an excess of unlabeled methionine. Analysis of total [3H]RNA samples showed that in control PRY105 cells, the pre-rRNAs were rapidly chased into 25 S and 18 S rRNAs, as expected. In contrast, in PRY106 cells expressing only the unphosphorylatable Ala-mutant Tif6p, the formation of 25 S rRNA was significantly inhibited without affecting the level of 18 S rRNA (Fig. 7B). Taken together, the experiments presented in Fig. 7, A and B, suggest strongly that Hrr25p-mediated phosphorylation plays an essential role for the optimal formation of both mature 25 S rRNA and 18 S rRNA.

**DISCUSSION**

Ribosome biogenesis in eukaryotic cells is a complex process that occurs primarily in the nucleolus where four ribosomal rRNAs are formed, modified, and processed during their assembly with 78 (in yeast) and 79 (in mammals) ribosomal proteins into mature 40 S and 60 S ribosomal subunits (31, 32). In *S. cerevisiae*, where the process has been best characterized, the 18 S rRNA of the 40 S subunit and the 25 S and 5.8 S rRNAs of the 60 S subunit are transcribed from a 9.1-kilobase rDNA transcription unit by RNA polymerase I as a single large precursor RNA known as the 35 S pre-ribosomal RNA (pre-rRNA) (see Fig. 6 for RNA processing intermediates). Immediately after synthesis of this pre-rRNA, many ribosomal proteins as well as a large number of transacting non-ribosomal proteins associate with the 35 S pre-rRNA to form the 90 S ribonucleoprotein particle. These transacting non-ribosomal proteins are required for pre-rRNA processing, pre-rRNA modification, and ribosome assembly. Tif6p, the yeast homologue of mammalian eIF6, is one of these essential 60 S ribosomal assembly proteins that associates with the 66 S pre-ribosomal particles in the nucleolus (7, 10) and is required for the processing of 27 SB pre-rRNA to the mature 25 S and 5.8 S rRNAs leading to formation of mature 60 S ribosomal particles (11).

In this study we show that Hrr25p, an isoform of yeast CK I, phosphorylates Tif6p at a single tryptic peptide both in vitro and in vivo. This peptide contains the only known CK I phosphorylation motif (D/E)nXXT located at serine residue 174 (Fig. 4). Of the isoforms of yeast CK I we analyzed, only Hrr25p can efficiently phosphorylate Tif6p (Fig. 2). Depletion of Hrr25p from yeast cells by a conditional mutant yeast strain confirms that Hrr25p is the kinase responsible for Tif6p phosphorylation in vivo (Fig. 3). Phosphopeptide mapping and mass spectrometric analysis show that Ser-174 is the major site of phosphorylation of Tif6p by Hrr25p (Fig. 4). These results are in agreement with our previous finding that mutation of Tif6p at Ser-174 to alanine drastically reduced phosphorylation (>75%).
and caused loss of cell growth and viability (11). When both Ser-174 and Ser-175 were mutated to alanine, phosphorylation of Tif6p was completely abolished (11).

In recent reports (27, 28), Hrr25p has been shown to stably associate with both the 90 S and the pre-40 S particles. The bound Hrr25p appears to phosphorylate two pre-40 S ribosomal assembly proteins, Enp1p and Ltv1p, as well as a 40 S ribosomal protein Rps3p in the pre-40 S ribosomal particles (28). Phosphorylation of these proteins appears to be required for the maturation of the pre-40 S particles. As discussed before, Tif6p is an essential 60 S ribosome assembly protein that associates with the 66 S pre-ribosomal particles. Analysis of the Tif6p-associated RNA components obtained by affinity purification of lysates of yeast cells expressing TAP-tagged Tif6p showed that Tif6p primarily associates with the two late 66 S pre-ribosomal particles (Fig. 6). Moreover, when we investigated the interaction between Tif6p and Hrr25p by coinmunoprecipitation, a small fraction of cellular Hrr25p was found to be associated with the 66 S pre-ribosomal particles to which Tif6p was also bound (Fig. 6). The presence of both Hrr25p and Tif6p on the same 66 S pre-ribosomal particles suggests that phosphorylation of Tif6p may occur on the 66 S preribosomal particles.

An important question that emerges is whether phosphorylation of Tif6p plays a role in the processing of 35 S pre-rRNA to form mature 18 S, 25 S, and 5.8 S rRNAs. Pulse-chase experiments presented to measure the rate of RNA processing reactions clearly show that depletion of Hrr25p from yeast cells results in severe inhibition in the processing of 35 S pre-rRNA to intermediate 27 S and 20 S pre-rRNAs. Subsequent processing of 20 S pre-rRNA to 18 S rRNA is also strongly inhibited, in agreement with the Northern analysis carried out by Schafer et al. (28). In contrast, Hrr25p-depleted yeast cells showed formation of 25 S rRNA, albeit at a much lower level than wild-type cells expressing Hrr25p. Additionally, in cells expressing only mutant Tif6p (S174A,S175A), where only Hrr25p-mediated phosphorylation of Tif6p is prevented without affecting phosphorylation of any other cellular proteins, formation of 25 S rRNA is also severely inhibited without affecting the formation of 18 S rRNA (Fig. 7B). Reports published from several laboratories (33, 34) have shown that Tif6p is required for nuclear export of pre-60 S ribosomal particles for final maturation in the cytoplasm. Thus, the possibility exists that lack of phosphorylation of Tif6p affects nuclear export of Tif6p-bound pre-60 S ribosomal particles. This results in inhibition of nucleocytoplasmic recycling of Tif6p that is presumably required for continued nucleolar 60 S biogenesis.

Both mammalian and yeast cells contain multiple isoforms of CK I that associate with different cellular compartments (12). These isoforms have a highly conserved catalytic domain in their N- and C-terminal domains. These non-catalytic domains contribute to the discrete cellular localization of each isoform and are essential for their respective functions (12). Many of the mammalian isoforms of CK I have been shown to be involved in specific regulatory functions, e.g. during embryonic morphogenesis (35), Wnt signaling pathway (36, 37), mRNA metabolism (38), cell cycle regulation (39), and circadian rhythm in Drosophila (40). Of the four isoforms of CK I in yeast, Hrr25p has emerged as a major regulatory kinase affecting a number of discrete biological processes. This kinase has been shown to be involved in transcriptional response to DNA damage and repair (41, 42), vesicle budding in the secretory pathway from the endoplasmic reticulum (43), cell survival during stress response through phosphorylation of stress-responsive transcription factor, Crz1p (16), and monopolar attachment of sister kinetochores at meiosis I (44). We now show that Hrr25p is also responsible for phosphorylation of Tif6p, an essential 60 S ribosomal biogenesis protein. This observation along with that of Schafer et al. (28) showing Hrr25p also mediates phosphorylation of several proteins of the pre-40 S ribosomal particles suggests that Hrr25p plays an important dual role in the biogenesis of both 40 S and 60 S ribosomal subunits. It is tempting to speculate that the constitutively active kinase Hrr25p might play a pivotal role in coordinating the process of 60 S and 40 S ribosome biogenesis in yeast.

Acknowledgments—We are indebted to Dr. Michael C. Keogh of Albert Einstein College of Medicine for critically reading the manuscript and for many helpful suggestions during the course of this work. We also thank Dr. Robyn Moir, Dr. Neelam Desai, and Dr. Ian Willis of Albert Einstein College of Medicine for considerable help in the preparation of yeast nuclear and cytoplasmic extracts. Finally, we are grateful to Dr. Jonathan Warner of Albert Einstein College of Medicine for many helpful discussions during the course of our work.

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