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Yeast Asc1p and Mammalian RACK1 Are Functionally Orthologous Core 40S Ribosomal Proteins That Repress Gene Expression

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Translation of mRNA into protein is a fundamental step in eukaryotic gene expression requiring the large (60S) and small (40S) ribosome subunits and associated proteins. By modern proteomic approaches, we previously identified a novel 40S-associated protein named Asc1p in budding yeast and RACK1 in mammals. The goals of this study were to establish Asc1p or RACK1 as a core conserved eukaryotic ribosomal protein and to determine the role of Asc1p or RACK1 in translational control. We provide biochemical, evolutionary, genetic, and functional evidence showing that Asc1p or RACK1 is indeed a conserved core component of the eukaryotic ribosome. We also show that purified Asc1p-deficient ribosomes have increased translational activity compared to that of wild-type yeast ribosomes. Further, we demonstrate that *asc1*Δ null strains have increased levels of specific proteins in vivo and that this molecular phenotype is complemented by either Asc1p or RACK1. Our data suggest that one of Asc1p's or RACK1's functions is to repress gene expression.

The eukaryotic 80S ribosome, consisting of small (40S) and large (60S) subunits, is the catalytic and regulatory macromolecular complex responsible for the decoding of mRNA into polypeptides. Together, the small and large ribosomal subunits contain the 18S, 28S (25S in yeast), 5.8S, and 5S rRNAs along with a large number of proteins. The structure and function of the ribosome have been extensively studied for decades, and a 15 Å cryoelectron microscopic map of the yeast ribosome is available (51). While the catalytic activity of the ribosome's 28S rRNA in peptide bond formation is well established, the function and regulatory activity of the ribosomal proteins are still largely unknown (12).

Advanced mass spectrometry (MS) analysis has facilitated the identification of novel ribosomal proteins. In a proteomic screen of the *Saccharomyces cerevisiae* 40S, 60S, and 80S components, we identified a novel component of the 40S and 80S subunits, Asc1p (*ASC1*, YMR116C, *CPC2*, *BEL1*), which remains associated with the small subunit in the presence of 1 M KCl (34). Under these stringent conditions, transient translation factors and ribosome biogenesis factors present in lower salt concentrations are shed from the 40S component. In these experiments, Asc1p was present at a concentration equimolar to that of the other ribosomal proteins (34). By established criteria for defining ribosomal proteins, Asc1p can be classified as a novel core 40S ribosomal component (31). We also demonstrated that RACK1 (receptor for activated C kinase 1), a protein with 52% sequence identity to Asc1p, is localized to the 40S and 80S components and polysomes in human cells (34). These observations have been confirmed by several other studies (2, 4, 6, 26, 50).

RACK1 was originally identified as a protein with sequence similarity to the guanine nucleotide-binding protein β subunit and other proteins containing Trp-Asp (WD) repeat domains (20). RACK1 was later shown to associate both in vitro and in vivo with activated protein kinase CβII (PKCβII) (46) and is hypothesized to function as an anchoring protein that localizes activated PKC to the insoluble cell fraction. A plethora of independent studies have attempted to define the molecular function of RACK1. Yeast two-hybrid and coimmunoprecipitation methods show RACK1 interacting with a large number of cellular proteins with roles in signal transduction (5, 9, 10, 15, 17, 21, 22, 29, 30, 32, 33, 36, 38, 41–43, 56, 62). Because of its apparent ability to interact with a number of signaling molecules, RACK1 is perceived to play a crucial role in a multitude of biological processes.

Consistent with our previous results showing association of Asc1p or RACK1 with the ribosome, more recent studies have implicated *ASC1* and RACK1 in translational control. General translational inhibition occurs upon amino acid starvation when the eIF2α kinase Gcn2p is activated (11). Yeast strains deficient in Gcn2p fail to initiate the amino acid starvation response. An *asc1* mutation has been shown to restore the amino acid starvation response in a *gcn2*Δ mutant strain (24). Further, *Schizosaccharomyces pombe cpc2*Δ (*asc1*Δ) null mutants appear to have a subset of genes that are transcriptionally and translationally repressed relative to those of the wild type (50). The *cpc2*Δ (*asc1*Δ) null strains were also sensitive to puromycin, a drug that causes premature termination of translation, suggesting that translational fidelity has been reduced (50). Moreover, in mammalian COS cells, transient overexpression of RACK1 stimulated translation in vivo (4).

In this report, we provide biochemical, evolutionary, genetic, and functional data showing that Asc1p or RACK1 is a core 40S ribosomal protein in eukaryotes. Further, our results suggest that Asc1p and RACK1 function to repress gene expression. This novel expression phenotype provides insight into a potential regulatory mechanism in eukaryotic gene expression.

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MATERIALS AND METHODS

Plasmids and yeast strains. Strain construction, genetic manipulations, and yeast medium preparation were carried out by standard methods (49). To construct plasmid p*ASC1*, a 2.13-kb BamHI-XbaI yeast genomic fragment from lambda clone λPM5992 (ATCC 70652) containing the entire *ASC1* locus was cloned into the BamHI and XbaI sites of pRS416 (8, 40, 44). Plasmid pME1867 (renamed pRACK1 in these studies), containing the rat cDNA for RACK1 expressed under the control of the *ASC1* promoter and terminator sequences in a pRS316 vector backbone, was a gift from Gerhard Braus (24). To construct plasmid pET100-*ASC1*, PCR primers A-ASC1 (CACCATGGCATCTAACGA AGTTTTAG), B-ASC1 (TTAAGTTCCAAGCCTTAACCATTTTGTCGTTA CCGGC), C-ASC1 (ACAAAATGGTTAAGGCTTGGAACTTAAACCAATT CC), and D-ASC1 (TTAGTTAGCAGTCATAACTTGCC) were used in a crossover PCR to amplify an intronless *ASC1* DNA fragment encoding the complete Asc1p protein (6). The intronless *ASC1* fragment was cloned into the pET100/D-TOPO bacterial expression vector (Invitrogen) to create plasmid pET100-*ASC1* encoding an N-terminally tagged His_6::*ASC1* fusion protein expressed from a T7 promoter. To construct plasmid p426-GPD::His_6-*ASC1*, a Klenow-treated 1.1-kb NdeI-SacI fragment from plasmid pET100-*ASC1* containing the His_6-*ASC1* fusion was cloned into the SmaI site of p426GPD (39). All constructs were confirmed by DNA sequencing.

The yeast strains used were BY4743 (*MAT***a**/*MAT*α *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/*leu2*Δ*0 met15*Δ*0*/*MET15 LYS2*/*lys2*Δ*0 ura3*Δ*0*/*ura3*Δ*0*) (60), YDM36556 (*MAT***a**/*MAT*α *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/*leu2*Δ*0 met15*Δ*0*/*MET15 LYS2*/*lys2*Δ*0 ura3*Δ*0*/*ura3*Δ*0* Δ*asc1*::Kan^r/Δ*asc1*::Kan^r) (60), AL150 (YDM36556 with pRS416), AL156 (YDM36556 with p*ASC1*), AL141 (YDM36556 with pRACK1), AL190 (BY4743 with pRS316), AL191 (BY4743 with p*ASC1*), AL140 (BY4743 with pRACK1), AL143 (YDM36556 with p426-GPD::His_6-*ASC1*), AL146 (YDM36556 with pYES-DEST52), AL103 (*MAT***a** *his7 ura3-52*), AL030 (*MAT***a** *his7 ura3-52 asc1*Δ) (34), AL185 (YDM36556 plus p180 [GCN4 5′ untranslated region {UTR}-*lacZ* reporter plasmid]) (23), and AL183 (BY4743 with p180). Strains containing chromosomal deletions of *asc1* were confirmed by PCR of yeast genomic DNA, PCR of yeast cDNA, and absence of Asc1p by two-dimensional (2D) gel electrophoresis.

Polysome analysis. Yeast cell extracts were prepared essentially as previously described (37). Briefly, yeast strains were grown in synthetic complete medium without uracil (SC−URA) to an optical density at 600 nm (OD_{600}) of 0.6, and 5 ml of cells was lysed with 0.5-mm glass beads in 250 μl of lysis buffer (10 mM Tris-HCl [pH 8.0] 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 200 U of RNasin [Promega] per ml). Extracts were centrifuged in a microcentrifuge for 1 min at 20,000 × *g*. Supernatants were supplemented with 250 μl of 2× translation stop buffer (20 mM dithiothreitol [DTT], 665 μg of heparin per ml, 150 μg of cycloheximide per ml) and 1 tablet of mini-Complete protease inhibitor (Roche) per 5 ml of buffer. The supplemented extracts were centrifuged for 5 min at 20,000 × *g*. Supernatants (200 μl) were gently layered on top of a 15 to 40% sucrose gradient cast in 10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM DTT, 100 μg of cycloheximide per ml, and 0.5 mg of heparin per ml. Gradients were centrifuged in a Beckman tabletop ultracentrifuge (TlS 55) for 75 min at 50,000 rpm. Fractions were collected from the top of the gradients and partitioned for RNA isolation, Western blotting, or MS analysis (34, 37). RNA was isolated from sucrose gradient fractions and yeast strains, with TRI-reagent LS (Molecular Research Center Inc. [MRC]) in accordance with the manufacturer's protocol. RNA from gradient fractions was loaded and run on nondenaturing 1% agarose gels cast in 1× Tris-acetate-EDTA and stained with ethidium bromide.

Western analysis and direct analysis of large protein complexes (DALPC). Western analysis was performed on polysome profile fractions from *S. cerevisiae* cells, human HEK293 cells, mouse NT2 cells, and in vitro translation extracts from *S. cerevisiae* strains. Sucrose gradient fractions or in vitro translation extracts were mixed with Laemmli buffer, heated for 5 min at 100°C, loaded onto NuPAGE 10% Bis-Tris gels, and separated with 1× morpholinepropanesulfonic acid-sodium dodecyl sulfate running buffer (Invitrogen). For Western analysis, NuPAGE gels were transferred to nitrocellulose membranes and blocked overnight in Tris-buffered saline containing 0.1% Tween and 10% nonfat dry milk. Western blots were probed with either affinity-purified rabbit polyclonal antibodies to Asc1p generated against full-length recombinant His_6-tagged Asc1p (Bethyl Laboratories, Montgomery, Tex.), mouse RACK1 monoclonal antibodies (BD Biosciences), Aip1p polyclonal antibodies (45), or Rpl3p monoclonal antibodies (59). Western blots were washed three times in Tris-buffered saline containing 0.1% Tween and then incubated with the appropriate horseradish peroxidase-tagged secondary antibody (Promega). Blots were developed with ECL Plus reagent (Amersham-Pharmacia). Asc1p antibody specificity was confirmed by Western blotting of Asc1p positive control antigen and whole-cell lysates from wild-type and *asc1*Δ null yeast strains (data not shown). The MS approach termed DALPC was performed essentially as described with *Drosophila melanogaster* embryos and *C. elegans* strain N2 pooled ribosomal and nonribosomal fractions (34, 47).

Genetic complementation of yeast *asc1*Δ mutant strains. Yeast strains were grown in either SC−URA or SC+URA to an OD_{600} of 0.6. Cells were counted with a hemocytometer, adjusted to the same concentration, and serially diluted (10-fold). Five microliters of each 10-fold serial dilution (10^8 to 10^5) was spotted onto SC−URA plates. The plates were incubated at 30 or 37°C for 72 h and photographed.

In vitro translation assays. To prepare translation extracts, 2 liters of yeast was grown in yeast extract-peptone-dextrose to an OD_{600} of 2. Cells were washed five times in ribosome buffer lacking protease inhibitors (30 mM HEPES [pH 7.4], 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM fresh DTT, 8.5% mannitol). After washing, 8 g of cells (wet weight) was lysed in 15 ml of ribosome buffer containing mini-Complete protease inhibitor (Roche) with 48 g of 0.5-mm glass beads. Cells were lysed in 50-ml sterile Falcon tubes by rigorous rocking in a 6-in. arc for 1-min intervals (five times) with 1-min intervals on ice between periods of rocking. Extracts were cleared by centrifugation twice at 20,000 × *g* for 10 min. Five milliliters of extract was loaded onto a 75-ml bed volume Sepharose G-25 column. The sample was fractioned with an isocratic buffer (ribosome buffer plus protease inhibitors) flowing at 0.5 ml/min. The flowthrough fractions (0.5 ml) with an OD_{260} of >90 were pooled and used for the in vitro translation assays (3).

Plasmid T3 lucpA, originally created by Peter Sarnow's laboratory (25), was kindly provided by Alan Sachs. T3 lucpA was purified with a QIAGEN miniprep and linearized with BamHI. The linearized plasmid was purified with a QIAquick PCR cleanup kit (QIAGEN). Capped luciferase mRNAs were synthesized with the Amplicap T3 high-yield message maker kit (Epicentre) with purified, linearized, T3 lucpA DNA as the template. The capped luciferase mRNAs were purified prior to in vitro translation with RNeasy spin columns (QIAGEN). Uncapped luciferase mRNA was purchased from Promega. Total RNA from wild-type yeast strain BY4743 grown to an OD_{600} of 1.0 was isolated with TRI-reagent (MRC). Following isolation of total RNA, poly(A)$^+$ mRNAs were isolated with an Oligotex mRNA isolation kit (QIAGEN). In vitro translation assays were conducted as described previously (54).

Assay for β-galactosidase activity. The p180 plasmid containing the 5′ UTR of GCN4 cloned in front of the *lacZ* gene was transformed into yeast strains BY4743 and YDM36556 (23). Strains were grown in SC−URA to an OD_{600} of 0.6. Cells were then pelleted by centrifugation at 9,000 × *g* for 5 min. Cells were lysed by bead beating in the 1× lysis buffer provided by the manufacturer (Promega). After lysis, extracts were centrifuged at 20,000 × *g* for 2 min. Following centrifugation, supernatants were assayed for β-galactosidase activity by the manufacturer's (Promega) protocol and measured for OD_{420} and OD_{280}. Relative β-galactosidase activity was standardized by dividing the β-galactosidase activity (OD_{420}) by the respective OD_{280} of each individual sample.

Purification of recombinant Asc1p. One liter of *Escherichia coli* strain BL21 containing plasmid pET100-*ASC1* was grown to an OD_{600} of 0.6 in Luria-Bertani medium. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside at 37°C for 1 h. Cells were harvested and lysed by sonication, and the recombinant protein was purified in accordance with the PROBOND kit (Invitrogen) native purification instructions. To prepare Asc1p protein for in vitro translation reactions, recombinant Asc1p protein was dialyzed exhaustively against ribosome buffer containing protease inhibitors.

2D difference gel electrophoresis (2D DIGE) analysis. One liter of each strain of yeast was grown in SC−URA to an OD_{600} of 0.6. Cells were harvested and lysed by bead beating in lysis buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 200 U of RNasin [Promega] per ml). After lysis, extracts were centrifuged at 20,000 × *g* for 10 min. Extracts were then partitioned for protein and mRNA analysis. Samples designated for 2D analysis were digested with RNase A and DNase and centrifuged again at 20,000 × *g* for 10 min prior to protein extraction. Three independent samples for each strain were prepared and analyzed.

2D DIGE analysis with a mixed-sample internal standard was carried out essentially as described previously (1, 13). Triplicate protein samples for each strain were individually labeled with either Cy3 or Cy5. To control for bias in the fluorescent dyes, we reversed the Cy3 and Cy5 dyes used to label the whole-cell extracts in one of each of the experimental replicates of the four strains being compared. The Cy2-labeled mixed-sample internal standard was composed of an equal portion of all 12 of the extracts used in the experiment. The Cy2 standard was used to normalize protein abundances across different gels and to control for gel-to-gel variation (1, 13). Equal amounts of Cy3-labeled sample, Cy5-labeled

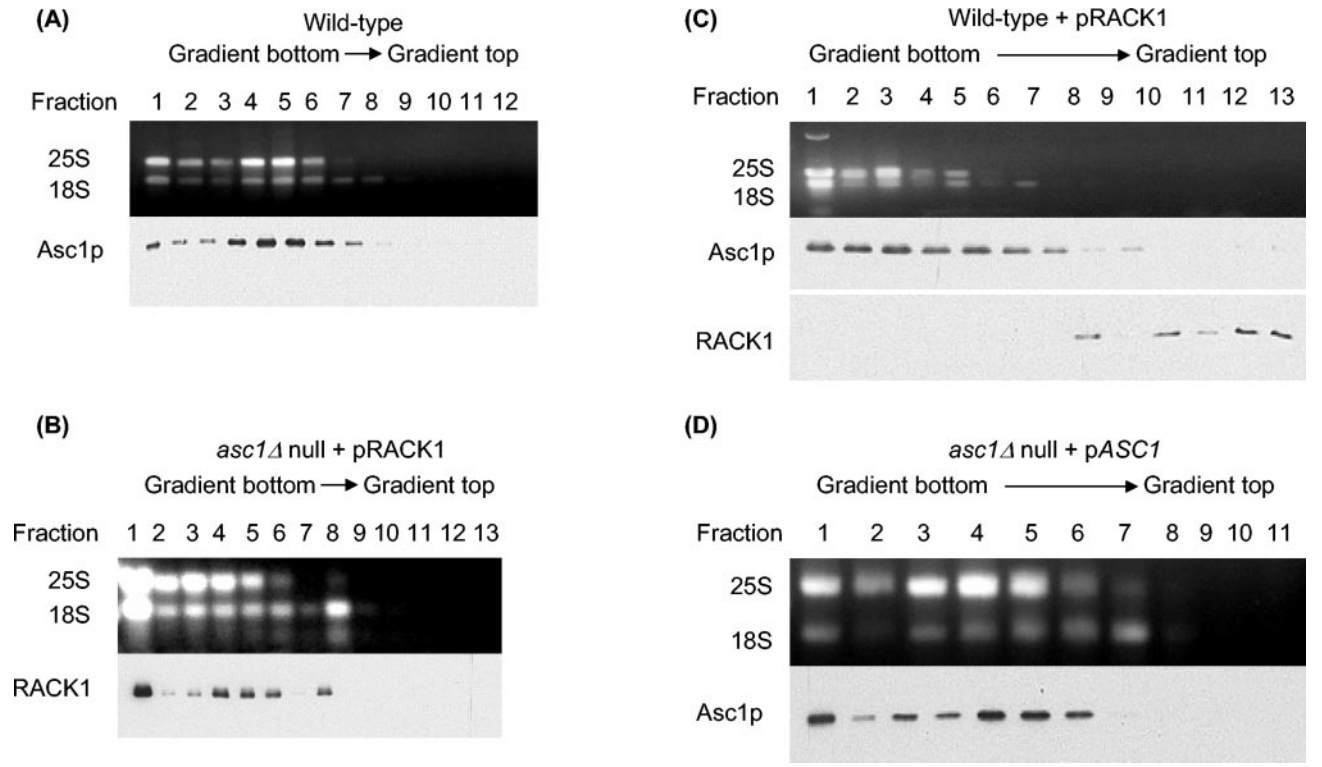


FIG. 1. Polysome profiles showing that Asc1p and RACK1 are biochemically orthologous ribosomal proteins. (A) Polysome profile showing that yeast Asc1p localizes to the polysome fractions and is absent in the nonribosomal fractions. Cell lysate from yeast wild-type strain AL190 was fractionated by sucrose gradient centrifugation, and fractions were collected. An aliquot of each fraction was analyzed by agarose gel electrophoresis to identify fractions with 25S and 18S rRNAs. Western analysis of the fractions with anti-Asc1p antibodies shows that Asc1p is in the ribosome fractions (lanes 1 to 8) and is absent from the nonribosomal fractions (lanes 9 to 12). (B) Polysome profile showing that RACK1 expressed in a yeast *asc1*Δ null strain localizes to the ribosomal fractions and is absent from the nonribosomal fractions. Cell lysate from yeast strain AL141 was fractionated by sucrose gradient centrifugation and analyzed as described for panel A except that anti-RACK1 antibody was used for Western analysis. (C) Polysome profile showing exclusion of RACK1 from ribosomal fractions in a wild-type yeast strain. Cell lysate from yeast strain AL140 was fractionated by sucrose gradient centrifugation and analyzed as described for panel A except that anti-RACK1 and anti-Asc1p antibodies were used in separate Western blot assays. (D) Polysome profile of an *asc1*Δ null strain complemented by expression of *ASC1*. Cell lysate from yeast strain AL156 was fractionated by sucrose gradient centrifugation and analyzed as described for panel A.

sample, and Cy2-internal control were mixed together and run on individual 2D gels. Triplicate Cy2-, Cy-3-, and Cy-5-labeled samples were then loaded onto a total of six 24-cm pH 4 to 7 immobilized pH gradient strips (Amersham Biosciences) and subjected to 2D gel electrophoresis with the IPGphor and DALT-twelve systems in accordance with the manufacturer's (Amersham Biosciences) protocols.

2D DIGE gels were scanned with a Typhoon 9410 variable-mode imager with the recommended mutually exclusive emission and excitation wavelengths for each Cy dye (Amersham Biosciences). 2D DIGE analysis was performed with DeCyder version 5.0 software (Amersham Biosciences), which uses a triple-codetection algorithm to generate the same protein spot-feature boundary for individual Cy2, Cy3, and Cy5 signals. The Cy3/Cy2 and Cy5/Cy2 ratios were then calculated and compared among the six DIGE gels, allowing for the application of Student *t* test statistical analyses to triplicate samples from all of the strains despite separation on different DIGE gels (1, 13).

In our analysis of in vivo protein levels, we considered 2D gel features to be a protein only if the spot was confirmed with both Cy labels and was present on every gel. Only proteins with ≥1.5-fold differences in abundance were considered significant. Differences in protein levels between wild-type and *asc1*Δ null samples were considered statistically significant only if the difference fell within the 95% confidence interval as determined by the Student *t* test.

Identification of 2D DIGE proteins. A SyproRuby (Molecular Probes) post-stain image (similarly acquired with a Typhoon 9410) was used to ensure accurate robotic protein excision for subsequent trypsin in-gel digestion with the ProSpot spot-handling workstation (Amersham Biosciences). Protein identifications were done by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS and TOF-TOF tandem MS with a Voyager 4700 MALDI-TOF–TOF mass spectrometer (Applied Biosystems). Protein identifications were based on the acquired mass spectral data combined with database interrogation by the MASCOT algorithm (Matrix Science).

Multiplex RT-PCR and real-time quantitative RT-PCR. Lysate-matched RNA extracts from the 2D DIGE protocol were analyzed for levels of mRNA transcripts encoding identified proteins. The extracts were treated with molecular biology grade DNase (Invitrogen) prior to reverse transcription (RT). RNA was reverse transcribed with oligo(dT) priming (Perkin-Elmer) and Superscript II (Invitrogen). Specific PCR primers were designed to the cDNA transcripts of interest with dsGENE software (Accelrys). Triplex PCR was performed by previously described protocols (18). The primer pairs used for RT-PCR were *TDH3* (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) (5′ TCTTCCATCTTC GATGCTGCCG and 5′ AGCCTTGGCAACGTGTTCAACC), *APE2* (5′ ACC AAAGGAAACCCAGGATGCG and 5′ AGCAGCTTTTTCAACGTCTGCG), *AIP1* (5′ CGTCCTTGTGAGCATTCAACGC and 5′ TCTTCGCGCAAAACC CTCGTAC), *DKA1* (5′ AAGCACGGCATTCTGGAGGATG and 5′ TCTTG GGGAACGTACGCATTCG), *ENO2* (5′ TAGAGCCGCTGCTGCTGA AAAG and 5′ TTGGAGCAACACCACCTTCGTC) *TPS1* (5′ TACAGGTTG CAGTGCCAAGTCG and 5′ ATTGTGCGGCACCTGTGAACTC), *ALD3* (5′ AAAGCTGCCAGGGCTGCTTTTG and 5′ TATTGAACTTGTCGACCGCC CC), and *CTT1* (5′ ACACCAGACACTGCAAGAGACC and 5′ TACGCGTT CATACTAGCCCACG). PCR samples were taken at cycles 17, 20, 23, 26, and 30. PCR products were run on 6% polyacrylamide gels cast in 0.5× Tris-borate-EDTA and stained with ethidium bromide.

For real-time RT-PCR, cDNA samples were prepared as described above. cDNAs were amplified in an IQ SYBR green supermix by the manufacturer's recommended protocol (Bio-Rad). Samples were analyzed in a Bio-Rad iCycler

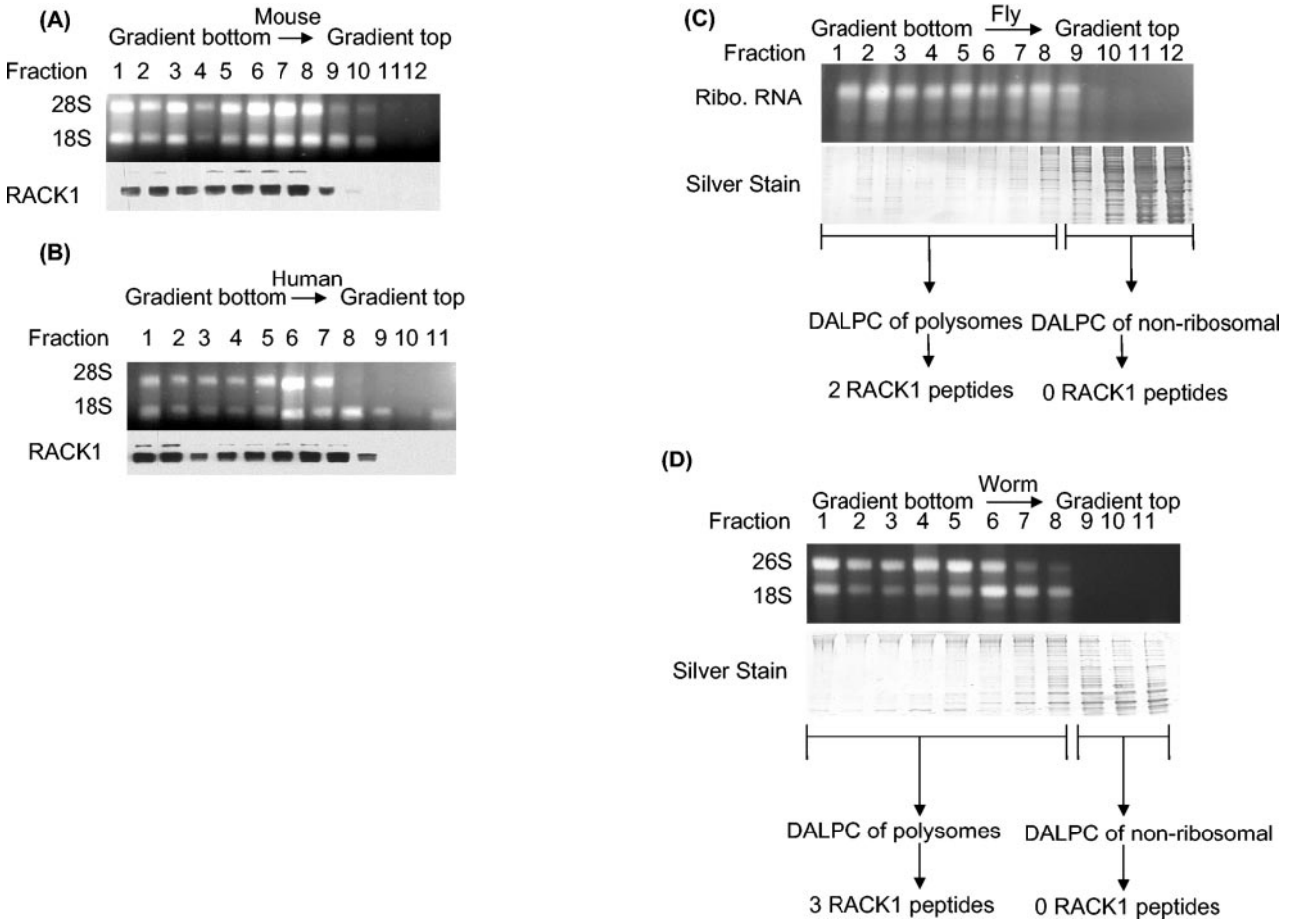


FIG. 2. Polysome profiling of RACK1 protein showing its polysomal localization in four eukaryotic species. (A) Mouse polysome profile showing that RACK1 localizes to the polysome fractions and is absent from the nonribosomal fractions. A cell lysate from mouse NT2 cells was fractionated by sucrose gradient centrifugation, and fractions were collected. An aliquot of each fraction was analyzed by agarose gel electrophoresis to show fractions with 28S and 18S rRNAs. Western analysis of the fractions with anti-RACK1 antibodies shows the distribution of RACK1. (B) Human polysome profile showing that RACK1 localizes to the polysome fractions and is absent from the nonribosomal fractions. A cell lysate from human HEK293 cells was fractionated by sucrose gradient centrifugation and analyzed as described for panel A. (C) DALPC analysis of the *Drosophila* polysome profile shows that the putative RACK1 ortholog (NP_477269) localizes to the polysome fractions and is absent from the nonribosomal fractions. Each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to visualize the amount of protein in each fraction. Polysome and nonribosomal fractions were separately pooled and digested with trypsin, and proteins were identified by the DALPC-MS approach (34, 47, 63). The *Drosophila* 28S and 18S rRNAs typically comigrate as a doublet (27). Ribo., ribosomal. (D) DALPC analysis of a nematode polysome profile shows that the putative worm RACK1 ortholog (NP_501859) localizes to the polysome fractions and is absent from the nonribosomal fractions. A lysate from wild-type worms was fractionated by sucrose gradient centrifugation, and fractions were collected. An aliquot of each fraction was analyzed by agarose gel electrophoresis to identify fractions with 28S and 18S rRNAs. Protein samples were analyzed as described for panel C.

with the following temperature cycle: 95°C for 10 s, 64°C for 1 min. PCR samples were cycled 32 times. To quantitate relative mRNA levels between yeast strains, mRNA levels were divided by the calculated *TDH3* (GAPDH) mRNA levels for each strain as a standard. For all of the cDNAs amplified, a stepwise melting curve protocol of 0.5°C was performed after PCR to confirm the presence of a single PCR product.

RESULTS

Mammalian RACK1 and *S. cerevisiae* Asc1p are orthologous ribosomal proteins. In a past study we showed that Asc1p localizes to the 40S and 80S subunits in *S. cerevisiae* and RACK1 localizes to the 40S subunit and polysomes in human HeLa cells (34). Sequence similarity and ribosome localization suggested that the two proteins are orthologous (http://linklab.mc.vanderbilt.edu). To further examine if RACK1 and Asc1p are biochemically orthologous ribosomal proteins, we performed a series of competitive and noncompetitive polysome profiling experiments with *S. cerevisiae*.

Polysome profiling results showed that in wild-type *S. cerevisiae*, Asc1p localized to ribosomal fractions but was absent from nonribosomal fractions (Fig. 1A). We reasoned that if RACK1 and Asc1p are orthologs, then in the absence of Asc1p, RACK1 should localize to ribosomes in yeast. When we expressed rat RACK1 (99% identical to human RACK1) in an *asc1*Δ null yeast strain, we found that RACK1 localized to ribosomal fractions in a polysome profile but was absent from nonribosomal fractions (Fig. 1B). This result indicated that RACK1 associated with the yeast ribosome similarly to Asc1p

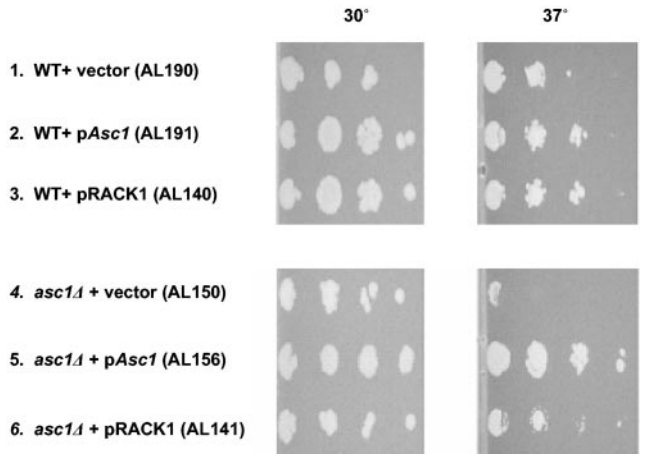


FIG. 3. Genetic complementation of the temperature-sensitive growth defect in a yeast *asc1*Δ null strain by *ASC1* and RACK1. The indicated yeast strains were grown to logarithmic phase in liquid medium, spotted in a dilution series onto SC−URA plates, grown at two different temperatures for 72 h, and then photographed. Strains 1 to 3 are wild-type (WT) controls. Experiments with strains 4 to 6 were performed in the *asc1*Δ null background. The genotypes of the strains are described in Materials and Methods.

and therefore might perform similar functions within the ribosome. Interestingly, when we expressed RACK1 in a wild-type yeast background we found that Asc1p was still included in ribosomal fractions but RACK1 was excluded (Fig. 1C). Because these results suggest that Asc1p and RACK1 compete for the same ribosomal position, we refer to this phenomenon as species-competitive-protein exclusion (compare Fig. 1B and C).

Localization of RACK1 to ribosomes is evolutionarily conserved in eukaryotes. Since the polysome profiling experiments suggested that Asc1p and RACK1 are biochemically orthologous ribosomal proteins, we wanted to investigate whether RACK1 is a conserved eukaryotic ribosomal protein. Alignment of yeast Asc1p with four putative orthologs from different species, *Caenorhabditis elegans* (NP_501859), *Drosophila melanogaster* (NP_477269), *Mus musculus* (BAA06185), and *Homo sapiens* (NP_006089), revealed a high level of sequence similarity among the different proteins (http://linklab.mc.vanderbilt.edu). To test if localization of these Asc1p-RACK1 orthologs is conserved among eukaryotic species, we performed polysome profiling of mouse, human, fly, and nematode extracts (Fig. 2A to D, respectively). By Western analysis, a RACK1 monoclonal antibody recognized a major and a minor band in the ribosomal fractions of *M. musculus* and *H. sapiens*. In both organisms, the RACK1 antibody failed to detect a protein in the nonribosomal fractions. Because the RACK1 antibody poorly recognized a cognate fly and worm protein, we analyzed *D. melanogaster* and *C. elegans* polysomes and nonribosomal fractions by the MS approach termed DALPC (34, 47, 63) (Fig. 2C and D). DALPC identified peptides derived from the putative othologous RACK1 proteins in the polysomal but not the nonribosomal fractions for both *D. melanogaster* and *C. elegans*. Together, our data show that in the four eukaryotes tested, *ASC1* and RACK1 were found in the ribosomal fractions and were absent from the nonribosomal fractions. Collectively, these results suggest that both the sequences and localization of *ASC1* and RACK1 to ribosomal fractions are conserved in eukaryotes.

***S. cerevisiae ASC1* and mammalian RACK1 are genetically orthologous.** Because polysome profiling experiments with yeast and higher eukaryotes indicated that Asc1p and RACK1 are biochemically conserved orthologous ribosomal proteins, we wanted to test to see if *ASC1* and RACK1 are genetically orthologous genes. We found that an *asc1*Δ null yeast strain had a temperature-sensitive growth defect (Fig. 3, line 4). With a plate dilution assay, we tested whether *CEN* plasmids expressing *ASC1* (p*ASC1*) or RACK1 (pRACK1) at the endogenous level of *ASC1* could complement the temperature-sensitive phenotype in an *asc1*Δ null strain. Complementation in the *asc1*Δ null strain was complete for *ASC1* and partial for RACK1 (Fig. 3, lines 5 and 6). These results are in excellent agreement with those of a previous study (24). Polysome profile analysis of the yeast *asc1*Δ null plus p*ASC1* complementing strain demonstrated that *ASC1* expression is restored and the complementing Asc1p protein is localized to the ribosomes (Fig. 1D). These data indicate that yeast *ASC1* and RACK1 are genetically orthologous.

Asc1p-deficient ribosomes have increased translational activity. Because our data indicated that RACK1 and Asc1p are conserved eukaryotic ribosomal proteins and earlier studies had implicated Asc1p in the amino acid starvation response, we wanted to determine the role of Asc1p or RACK1 in translation. To evaluate the activity of Asc1p in translation, we performed in vitro translation assays with extracts from wild-type and *asc1*Δ null yeast strains (54). In three independent experiments translating a capped and polyadenylated luciferase reporter mRNA, the *asc1*Δ null strain ribosomes had translational activity 3- to 10-fold higher than that of wild-type ribosomes (Fig. 4A and F). To show that the increased translational activity was not strain dependent, in vitro translation assays were performed with extracts from genetically independent wild-type and *asc1*Δ null strains (34). Similar to the earlier strains, the second *asc1*Δ null strain had higher in vitro translational activity than did the isogenic wild-type strain (Fig. 4B).

To verify that the *asc1*Δ null increased translational activity phenotype was independent of the reporter molecule, we translated uncapped luciferase, capped luciferase, wild-type poly(A)$^+$ mRNAs, and a GCN4 5′ UTR-*lacZ* reporter. We found that translational activity is elevated in the *asc1*Δ null strain compared to that in the wild-type, control strains (Fig. 4A through F).

In an attempt to modulate the higher translational activity of the ribosomes lacking Asc1p, we added recombinant Asc1p protein to our in vitro extracts (http://linklab.mc.vanderbilt.edu). Expression of the recombinant protein in *asc1*Δ null strains complemented the temperature-sensitive growth defect (http://linklab.mc.vanderbilt.edu). However, addition of exogenous Asc1p protein to the in vitro translation extracts failed to repress translational activity (Fig. 4F).

Since the recombinant protein failed to restore translational activity to wild-type levels, we sought to determine if our recombinant protein was added at wild-type stoichiometric levels. Western analysis of in vitro translation extracts showed the levels of exogenous Asc1p equal to or greater than that found in wild-type ribosome preparations (Fig. 4G). When relative

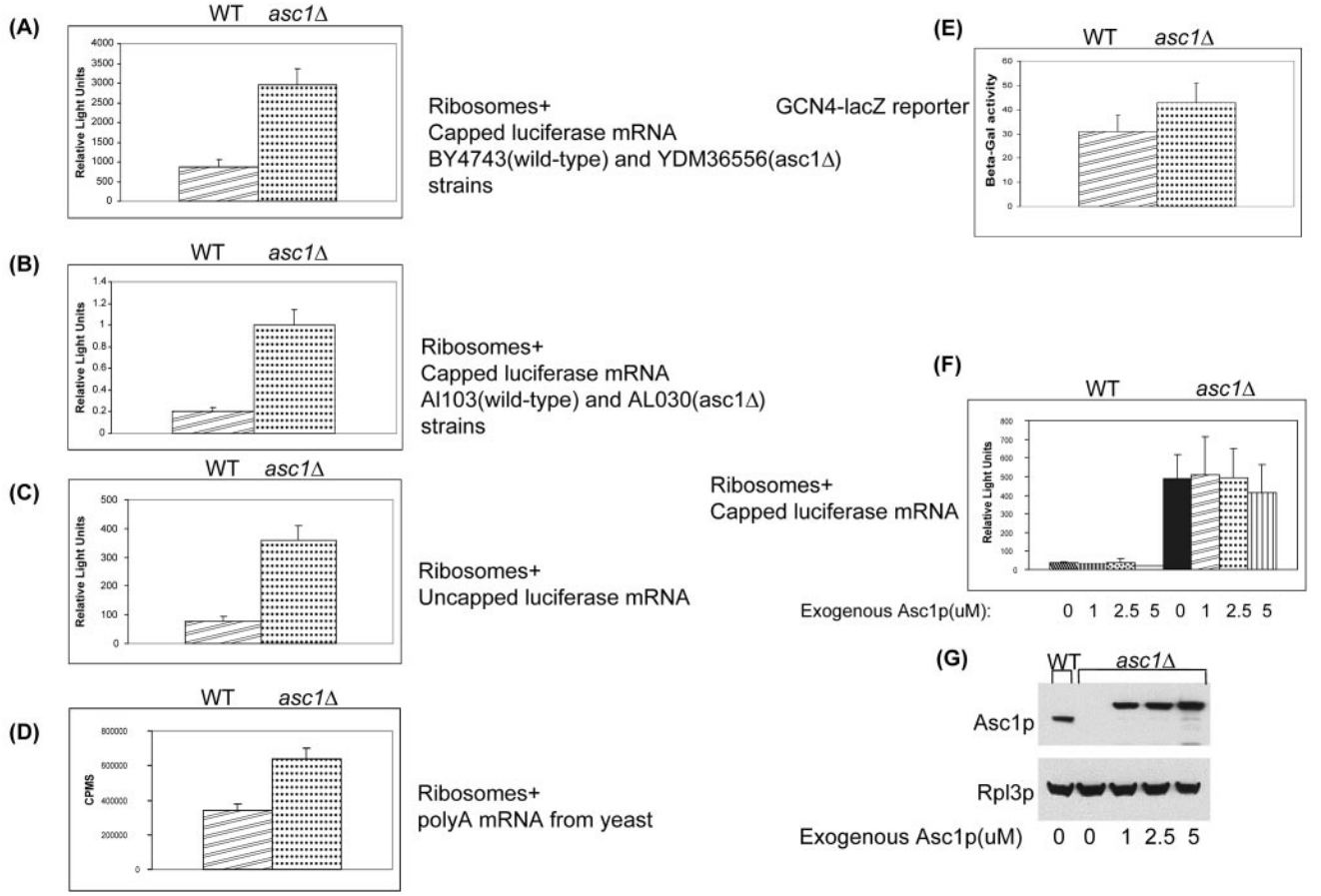
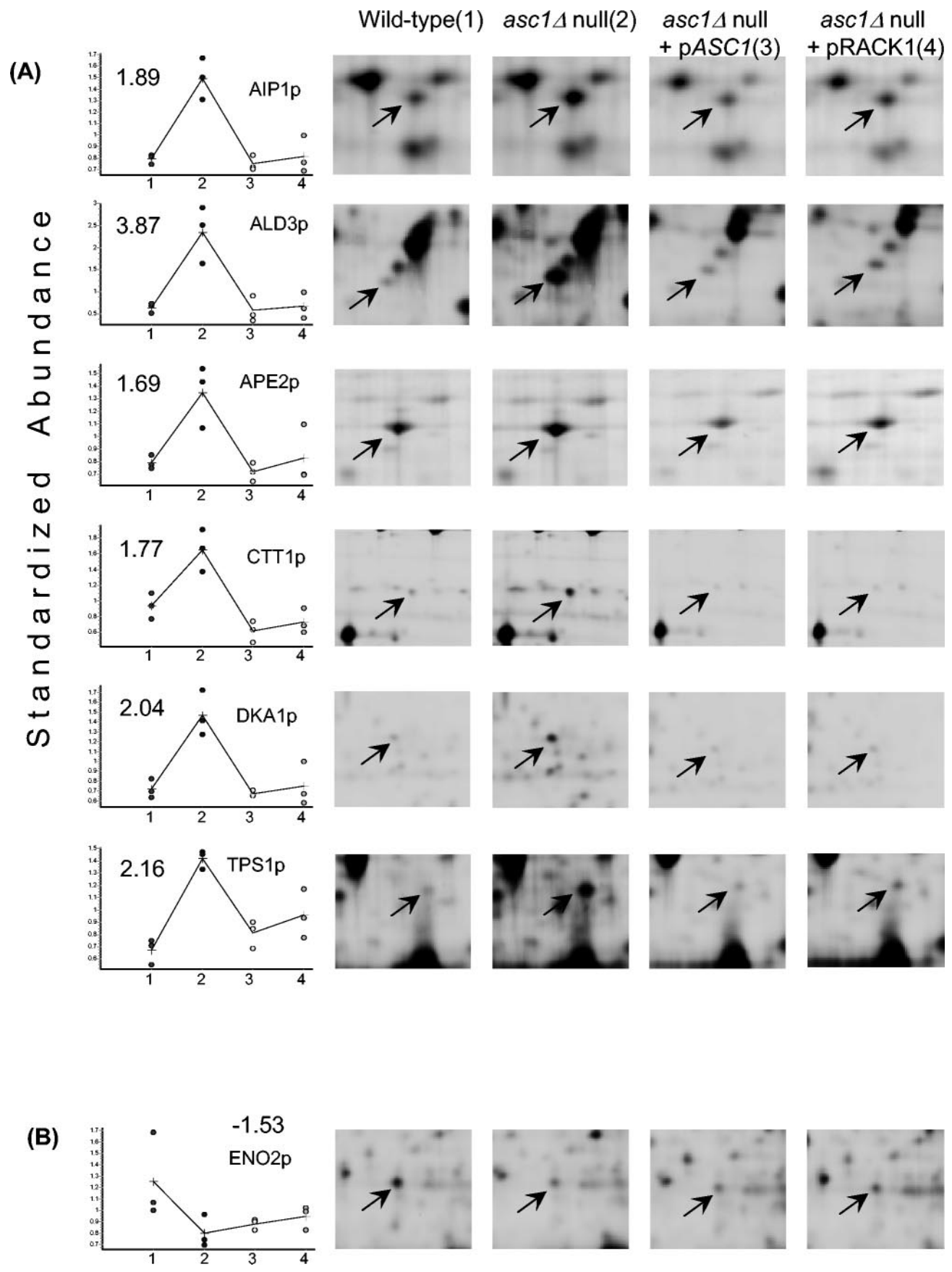


FIG. 4. Up-regulation of translational activity in *asc1*Δ extracts. (A) In vitro translation of capped and polyadenylated luciferase reporter mRNA with extracts prepared from wild-type (WT) BY4743 and isogenic *asc1*Δ null strain YDM36556. Translational activity was determined by measuring luminescence (relative light units) after 30 min of incubation at 26°C. Error bars indicate standard deviations. (B) In vitro translation of capped and polyadenylated luciferase reporter mRNA with extracts prepared from wild-type AL103 and isogenic *asc1*Δ null strain AL030. (C) In vitro translation of uncapped and polyadenylated luciferase reporter mRNA with extracts prepared from wild-type BY4743 and isogenic *asc1*Δ null strain YDM36556. (D) In vitro translation of poly(A)-enriched mRNAs with extracts prepared from wild-type BY4743 and isogenic *asc1*Δ null strain YDM36556. The extracts were incubated with whole wild-type yeast mRNA and [^{35}S]methionine. Translational activity was determined by measuring the counts per min after 30 min of incubation at 26°C. (E) In vivo analysis of GCN4 translation. Wild-type BY4743 and *asc1*Δ null YDM36556 strains were transformed with a GCN4 5′ UTR-*lacZ* reporter plasmid (p180) (23). Cells were grown in SC−URA to an OD_{600} of 0.6 and assayed for β-galactosidase activity as described in Materials and Methods. (F) In vitro translation of capped and polyadenylated luciferase reporter mRNA in the presence of an increasing concentration of recombinant Asc1p protein. Protein extracts were prepared from wild-type BY4743 and isogenic *asc1*Δ null strain YDM36556. The extracts were incubated with luciferase mRNA and the indicated amounts of recombinant Asc1p protein. Translational activity was determined by measuring luminescence (relative light units) after 30 min of incubation at 26°C. (G) Western analysis of in vitro extracts with anti-Asc1p and anti-Rpl3p antibodies. As a loading control for the in vitro translation assay, Asc1p and Rpl3p levels were measured. Poly-His-tagged recombinant Asc1p migrates more slowly than endogenous Asc1p. Rpl3p levels suggest that 60S subunit levels were similar between wild-type and *asc1*Δ translation extracts.

levels of Rpl3p protein were compared between extracts, the amounts of ribosomes appeared to be similar (Fig. 4G). These data suggest that equal amounts of ribosomes were present in each extract for translation. Collectively, these data show that ribosomes lacking Asc1p have increased (derepressed) translation activity, suggesting that Asc1p acts as a translational repressor.

Asc1p or RACK1 functionally complements increased protein levels in *asc1*Δ null strains. Because the in vitro data above suggested that Asc1p functions as a translational repressor, we sought to analyze in vivo changes in protein levels among wild-type, *asc1*Δ null, *asc1*Δ null plus p*ASC1*, and *asc1*Δ null plus pRACK1 complemented strains. We used 2D DIGE to quantify any changes in the in vivo protein levels for a large population of proteins (1, 13). In the 2D DIGE experiments, whole-cell lysates were prepared from three independent cultures for each of the four strains described above (i.e., 12 independent samples). The lysates were labeled prior to electrophoresis with the spectrally resolvable fluorescent dye Cy3 or Cy5. To normalize for protein abundance differences across multiple 2D gels, a mixed internal standard pool containing equal amounts of the experimental samples was labeled with a Cy2 fluorescent dye. The pooled standard represented the average of all of the samples being compared. Independent Cy2, Cy3, and Cy5 samples were mixed and resolved on the same 2D gels. As a consequence, each gel contained an image


(A)
Wild-type(1)
asc1Δ null(2)
asc1Δ null + pASC1(3)
asc1Δ null + pRACK1(4)
Standardized Abundance
1.89
AIP1p
3.87
ALD3p
1.69
APE2p
1.77
CTT1p
2.04
DKA1p
2.16
TPS1p
(B)
-1.53
ENO2p

with a highly similar spot pattern, simplifying and improving the confidence of inter-gel spot matching and quantification (1). To control for bias in the fluorescent dyes, we reversed the Cy3 and Cy5 dyes used to label the whole-cell extracts in one of the experimental triplicates of the four strains being compared.

In our analysis of in vivo protein levels, we interpreted a 2D gel feature as a protein only if the spot was confirmed with both the Cy3 and Cy5 labels and was present on every gel. The total number of proteins analyzed (approximately 1,500) included multiple isoforms of some individual proteins. Only proteins with ≥1.5-fold differences in abundance were considered significant and included in our analysis. In addition, differences in protein levels between wild-type and *asc1*Δ null samples were considered statistically significant only if the difference fell within the 95% confidence interval as determined by the Student *t* test.

2D DIGE analysis indicated that of the ~1,500 yeast proteins detected, 27 are ≥1.5-fold more abundant in the *asc1*Δ null strain than in the wild type ($P < 0.05$) (Fig. 5A and http://linklab.mc.vanderbilt.edu). Of these 27 elevated proteins, the differences in abundance between the wild-type and *asc1*Δ null strains ranged from 1.5- to 4.44-fold. Six of the 27 up-regulated had sufficient material for unambiguous identification by MS (Fig. 5A). For *AIP1* up-regulated in the *asc1*Δ null strain, we performed Western analysis with the same extracts used for the 2D DIGE experiments. The relative abundance of Aip1p from the four strains was consistent with results from the 2D DIGE experiments (http://linklab.mc.vanderbilt.edu). Only 3 of the 1,500 proteins demonstrated statistically significant ($P < 0.05$) down-regulation in the *asc1*Δ null strain in comparison to the wild-type strain (Fig. 5B and http://linklab.mc.vanderbilt.edu). One of the three proteins had sufficient material for identification by MS (Fig. 5B). Both the *asc1*Δ null plus p*ASC1* and *asc1*Δ null plus pRACK1 strains restored elevated protein levels back to wild-type levels for 24 of 27 of the up-regulated proteins (Fig. 5A and http://linklab.mc.vanderbilt.edu). In contrast, levels of the three down-regulated proteins were not complemented by the *asc1*Δ null plus p*ASC1* or the *asc1*Δ null plus pRACK1 strain (Fig. 5B and http://linklab.mc.vanderbilt.edu). These results are consistent with our in vitro experiments suggesting a repressive role for Asc1p in translation. Additionally, these data provide further evidence that Asc1p and RACK1 are functionally orthologous ribosomal proteins.

To distinguish between transcriptional and posttranscriptional regulation of gene expression for the seven identified proteins, we compared 2D DIGE protein abundance data with the levels of mRNAs from the four strains. The levels of mRNAs encoding six up-regulated proteins (AIP1p, ALD3p, APE2p, CTT1p, DKA1p, and TPS1p) and one down-regulated protein (ENO2p) were measured in the wild-type, *asc1*Δ null, and complemented strains by multiplex RT-PCR and real-time RT-PCR. By triplex RT-PCR we observed no apparent difference in transcript levels for any of the seven genes analyzed (Fig. 6A). By real-time quantitative PCR, the average differences in *AIP1*, *ALD3*, *APE2*, *CTT1*, *DKA1*, and *TPS1* transcript abundance were variable but not statistically significantly different in the *asc1*Δ null strain relative to those in the wild-type and complemented strains ($P < 0.05$) (Fig. 6B). However, ENO2 transcript levels were down-regulated in the *asc1*Δ null strain relative to those in the wild type ($P < 0.05$) (Fig. 6A). Collectively, these results support a negative role for Asc1p or RACK1 in gene expression.

DISCUSSION

RACK1 or Asc1p is the 33rd ribosomal protein of the small (40S) eukaryotic subunit. In the classical experiments of Kruiswijk and Planta, proteins were considered core ribosomal components if they remained associated in the presence of 0.5 M KCl (31). Asc1p fulfills this requirement by associating with the ribosome in the presence of 1 M KCl (34). Localization of Asc1p or RACK1 to the 40S, 80S, and polysomal fractions has been observed by several independent studies (2, 4, 6, 26, 34, 50). Here we have demonstrated the localization of Asc1p and RACK1 to polysomes in five different eukaryotic species. Further, we demonstrate that yeast Asc1p and mammalian RACK1 compete for localization to the yeast ribosome. Moreover, we show that RACK1 functionally complements the phenotype of an *asc1*Δ null mutant. These data, taken together with the results of other studies, strongly suggest that Asc1p and RACK1 are orthologous core ribosomal proteins. Consequently, we propose that a more appropriate name for yeast *ASC1* is *RPS33*, the 33rd ribosomal protein in the *S. cerevisiae* 40S small subunit. By corollary, RACK1 should be classified as a mammalian 40S core ribosomal protein.

ASC1 has several other features that are common to *S. cerevisiae* ribosomal genes. Although only a small percentage of *S. cerevisiae* genes contain an intron, 66% of the yeast ribosomal genes contain an intron. Two previous studies have shown that *ASC1* contains an intron (6, 24). The codon adap-

FIG. 5. In vivo changes in protein levels in *asc1*Δ null strains. (A) Up-regulated proteins in the *asc1*Δ null strain are complemented by either Asc1p or RACK1. Graphs of standardized protein abundance in wild-type (no. 1 on the *x* axis), *asc1*Δ null (no. 2 on the *x* axis), *asc1*Δ null plus p*ASC1* (no. 3 on the *x* axis), and *asc1*Δ null plus pRACK1 (no. 4 on the *x* axis) complemented strains are shown. Representative 2D gel images are shown as a reference for protein levels for each strain and show an arrow pointing to the Cy3- or Cy5-labeled protein identified. In the graphs, each dot indicates the standardized protein abundance of a specific protein for a given strain in a single independent experiment. Each dot was calculated by dividing the Cy3 or Cy5 density by the Cy2 density (internal standard) for the respective protein position. Values on the graphs indicate the fold differences in average standardized abundance between the *asc1*Δ null and wild-type strains. A plus sign marks the average standardized abundance for a given strain and is representative of three independent experiments. The black line connects the average standardized protein abundances with one another. (B) The down-regulated protein in the *asc1*Δ null strain is not complemented by either Asc1p or RACK1. The graph shows standardized protein abundances in the wild-type (no. 1 on the *x* axis), *asc1*Δ null (no. 2 on the *x* axis), and *asc1*Δ null plus p*ASC1* (no. 3 on the *x* axis) and *asc1*Δ null plus pRACK1 (no. 4 on the *x* axis) complemented strains. The graphs and 2D gel images are similar to those in panel A.

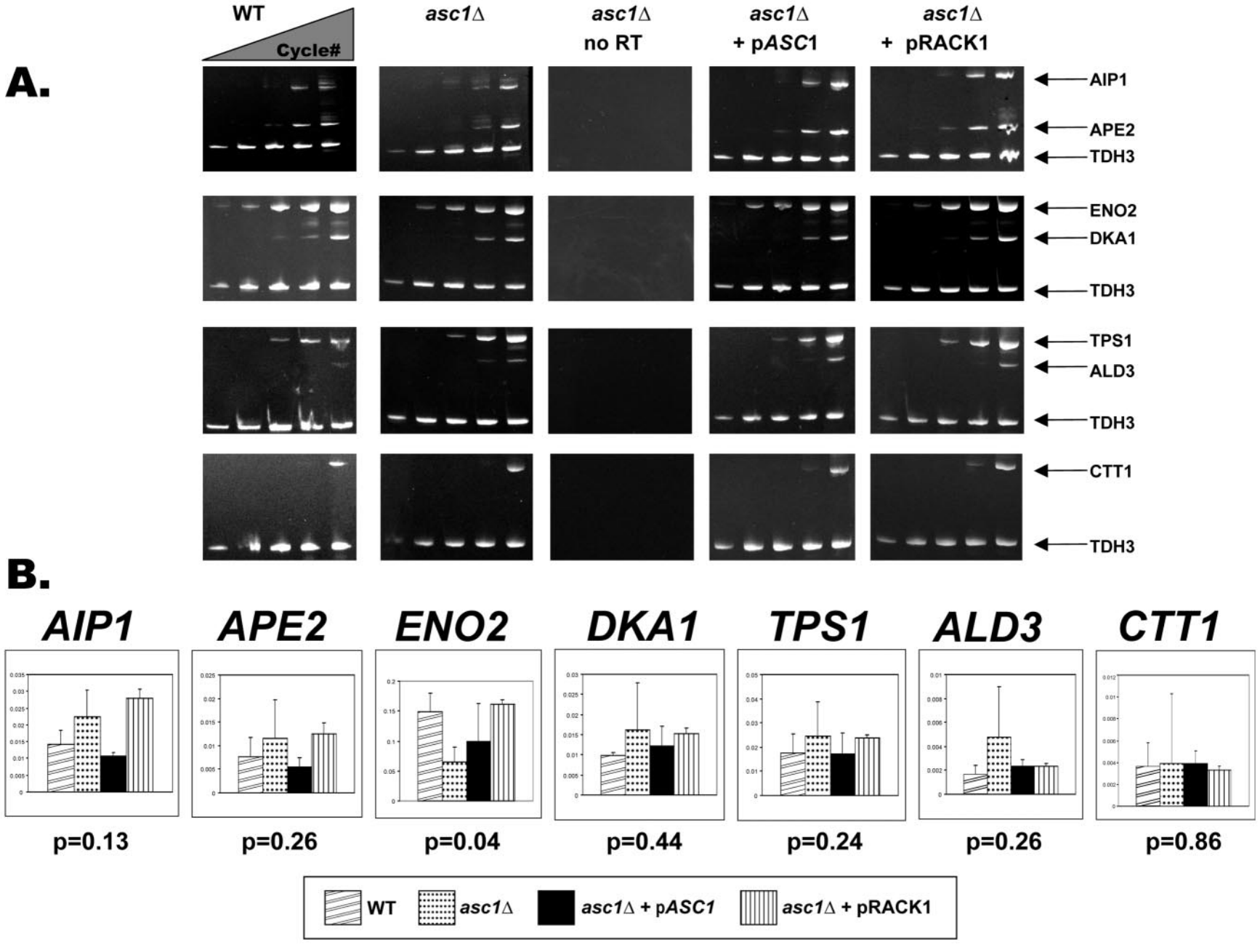

A.
WT
Cycle#
asc1Δ
asc1Δ no RT
asc1Δ + pASC1
asc1Δ + pRACK1
AIP1
APE2
TDH3
ENO2
DKA1
TDH3
TPS1
ALD3
TDH3
CTT1
TDH3
B.
AIP1
APE2
ENO2
DKA1
TPS1
ALD3
CTT1
p=0.13
p=0.26
p=0.04
p=0.44
p=0.24
p=0.26
p=0.86
WT
asc1Δ
asc1Δ + pASC1
asc1Δ + pRACK1

FIG. 6. Analysis of mRNA transcript levels in *asc1*Δ null, *asc1*Δ null with p*ASC1*, *asc1*Δ null with pRACK1, and wild-type strains. (A) Triplex RT-PCR of mRNA transcripts in *asc1*Δ null, *asc1*Δ null with p*ASC1*, *asc1*Δ null with pRACK1, and wild-type strains used for 2D DIGE analysis. As a negative control, *asc1*Δ null RNA was amplified with no reverse transcriptase. *AIP1*, *ALD3*, *APE2*, *CTT1*, *DKA1*, *ENO2*, and *TPS1* mRNA transcript abundances were measured by triplex semiquantitative RT-PCR with total RNA prepared from 2D DIGE experiments. cDNA transcripts were coamplified with *TDH3* (GAPDH) as the standard. One of three independent samples is shown here. PCR cycles 17, 20, 23, 26, and 30 are shown in order from left to right for each sample. (B) Quantification of mRNA levels in *asc1*Δ null strains. The graph shows the quantified levels of the mRNAs for the seven genes from the four different strains in panel A. Levels of transcripts for all seven proteins identified by MALDI-TOF were quantified by real-time PCR of cDNA transcripts. As a standard, mRNA transcript levels for each gene were divided by the *TDH3* (GAPDH) transcript levels in each sample. Error bars indicate the standard deviation of the mean. *P* values for each gene were determined through a Student *t* test comparing the calculated wild-type and *asc1*Δ null transcript levels.

tation index (CAI) is a measurement of the relative adaptiveness of the codon usage of a gene toward the codon usage of highly expressed genes (28, 48). CAIs range from 0 to 1, with higher values indicating a higher proportion of the most abundant codons. The average CAI for all yeast genes is 0.18, while the average CAI for ribosomal genes is 0.71. *ASC1* has a CAI of 0.77, suggesting that it is highly expressed. Several studies have measured the transcriptional expression of *ASC1* and found it to be expressed at levels equivalent to those of ribosomal genes (58, 61). A recent study measured the abundances of a large number of yeast proteins (19). The estimated abundances of ribosomal proteins ranged from 4.5×10^3 to 6.02×10^5 molecules per log-phase cell, with an average of 7.0×10^4 molecules per cell. Asc1p was found to be present at approximately 3.33×10^5 molecules per log-phase cell, which is in the same range as other ribosomal proteins (19). These results agreed with an earlier study showing that Asc1p is an abundant yeast protein that is highly enriched in ribosomal fractions (16). Asc1p is present at a concentration equimolar to that of the other ribosomal proteins (34). All of these reports indicate that *ASC1* is a highly expressed yeast gene with characteristics similar to those of other ribosomal genes.

***ASC1* or RACK1 exerts a repressive effect on protein synthesis.** We found that absence of yeast Asc1p resulted in elevated translational activity. However, this molecular phenotype could not be complemented in vitro by adding recombinant Asc1p. While it is possible the recombinant Asc1p fusion protein is not folded properly or lacks critical modified residues, the fusion protein does complement an *asc1*Δ null strain. Failure of a core eukaryotic ribosomal protein to complement when added exogenously is not surprising since eukaryotic ribosome assembly is thought to occur as a stepwise process in the nucleolus (14, 57). In contrast, translational initiation factors can be added exogenously to restore activity to wild-type levels in in vitro translation assays (7, 55).

To identify the molecular function of *ASC1* and *RACK1* in vivo, we used a quantitative global proteomic analysis of the wild-type, *asc1*Δ null, and yeast p*ASC1*-complemented and mammalian p*RACK1*-complemented strains. We reasoned that if Asc1p and RACK1 function as repressive ribosomal proteins, we might observe a global up-regulation of protein levels in *asc1*Δ null strains. Further, this up-regulation should be complemented by expression of yeast p*ASC1* or mammalian pRACK1 in an *asc1*Δ null background. Indeed, we found that *asc1*Δ null strains have elevated levels of some proteins, and this molecular phenotype can be complemented by either yeast p*ASC1* or mammalian pRACK1. The changes in protein levels appear to be independent of mRNA levels and therefore likely occur through a posttranscriptional mechanism.

The in vitro translation assays with various mRNA templates suggest that translation activity is globally increased for ribosomes lacking Asc1p. However, in vivo assessment of protein levels in *asc1*Δ null strains shows that only a specific population of proteins (27 of 1,500) is significantly up-regulated. Although in vitro translation assays suggested a 2- to 10-fold increase in translational activity for the *asc1*Δ null strain ribosomes, in vivo posttranscriptional analysis indicated that specific protein levels were up-regulated between 1.5- and 4.44-fold (compare Fig. 4 and 5). The in vitro translation assays are by nature an artificial environment for translating mRNAs. Therefore, a 1:1

correlation with the in vivo results should not be expected. Despite the observed discrepancy between in vitro and in vivo results, both assays suggest a general trend of increased translational activity for the *asc1*Δ null strain. Taking the results of both assays into consideration, we propose that Asc1p functions as a translational repressor.

Recent reports suggest that Asc1p and RACK1 function to stimulate eukaryotic translation (4, 50). We speculate that these observations arise from indirect phenomena initiated by the translational up-regulation of regulatory proteins. For example, in this study we observed a reduction of ENO2 protein and mRNA levels in the *asc1*Δ null strain (Fig. 6A and B). It is conceivable that this reduction in transcript levels arises from the translational up-regulation of a transcriptional repressor.

A previous study observed an increase in specific mRNA levels in *asc1*Δ null strains relative to those in wild-type strains (24). Interestingly, these up-regulated mRNAs in *asc1*Δ null strains are regulated by the transcription factor Gcn4p (24). However, in the same study *asc1*Δ null strains did not show translational up-regulation of GCN4. Here we observed up-regulation of *GCN4* translational activity relative to that of the wild type (Fig. 4E). Because a multiplicity of coactivators contributes to *GCN4*-mediated transcription, it is possible that specific mRNAs are elevated in *asc1*Δ null strains through the translational up-regulation of specific *GCN4* coactivators (53). Our results support a model in which Asc1p and RACK1 are core components of the 40S ribosomal subunit that modulate the translation of mRNAs. Because our results show that average mRNA levels are often higher, but not statistically significantly different, in *asc1*Δ null strains, it is possible that Asc1p and RACK1 play an indirect role in transcriptional repression.

A number of proteins have been reported to interact with RACK1 in mammalian systems. Although we have not addressed this question, we observe RACK1 in ribosomal fractions when analyzing mammalian species. It is possible that RACK1 dissociates from the ribosome to perform other functions. A precedent for such phenomena has recently been reported for ribosomal protein L13A (35). Several studies have reported an association of RACK1 with the βII isoform of PKC (46, 52). Interactions of RACK1 with signal transduction proteins may direct ribosomes to specific cellular locations where localized translation of proteins is required. Further experiments are required to test these different models.

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