Saccharomyces cerevisiae Rbg1 Protein and Its Binding Partner Gir2 Interact on Polyribosomes with Gcn1

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Rbg1 is a previously uncharacterized protein of Saccharomyces cerevisiae belonging to the Obg/CgtA subfamily of GTP-binding proteins whose members are involved in ribosome function in both prokaryotes and eukaryotes. We show here that Rbg1 specifically associates with translating ribosomes. In addition, in this study proteins were identified that interact with Rbg1 by yeast two-hybrid screening and include Tma46, Ygr250c, Yap1, and Gir2. Gir2 contains a G1 (Gcn2 and Impact) domain similar to that of Gcn2, an essential factor of the general amino acid control pathway required for overcoming amino acid shortage. Interestingly, we found that Gir2, like Gcn2, interacts with Gcn1 through its G1 domain, and overexpression of Gir2, under conditions mimicking amino acid starvation, resulted in inhibition of growth that could be reversed by Gcn2 co-overexpression. Moreover, we found that Gir2 also cofractionated with polyribosomes, and this fractionation pattern was partially dependent on the presence of Gcn1. Based on these findings, we conclude that Rbg1 and its interacting partner Gir2 associate with ribosomes, and their possible biological roles are discussed.

The Obg/CgtA subfamily is a conserved group of monomeric GTP-binding proteins found in the genomes of all organisms sequenced thus far. The evolutionary relationships of the GTPase superfamily suggested a role in ribosome function (6, 38). This prediction appears accurate, since all mitochondrial (8), nuclear (12, 28, 31), and prokaryotic (29, 39, 51, 59–61, 70) Obg/CgtA proteins examined thus far are associated with ribosomes. Moreover, these proteins are also involved in the assembly of the large ribosomal subunit (28, 29, 31, 37, 51, 55). Recently, it has become clear that, in addition to a role in the late assembly of the large ribosomal subunit, the bacterial Obg/CgtA proteins are also directly involved in stress response (30, 48, 58).

In Escherichia coli, amino acid starvation leads to uncharged tRNAs binding to the ribosomal acceptor site (A-site) in a codon-dependent manner (18) which is detected by RelA, a (p)ppGpp synthetase. The increase in (p)ppGpp levels leads to the “stringent response” that provides the cell with the regulatory means to control gene expression and thereby cope with starvation. The levels of (p)ppGpp are kept low in nutrient-rich media by SpoT, a bifunctional enzyme related to RelA that has both (p)ppGpp synthetase and hydrolase activity (19, 20, 71). The hydrolase activity of SpoT is inhibited under nutrient-limiting conditions, allowing intracellular (p)ppGpp levels to increase during the stringent response, as RelA produces (p)ppGpp. In E. coli and Vibrio cholerae, the GTP-binding protein CgtA (also called YhbZ or Obg) interacts with SpoT (48, 70) on the large ribosomal particle (30). Depletion of CgtA results in an increase in (p)ppGpp levels (30, 48), raising the possibility that during exponential growth CgtA directly inhibits the hydrolase activity of SpoT.

It has been proposed that, in Saccharomyces cerevisiae, amino acid starvation also leads to uncharged tRNAs binding to the A-site but that the output is not RelA synthesis of (p)ppGpp (21). The effector protein Gcn1 detects the uncharged RNA and relays the A-site occupancy information to the protein kinase Gcn2. Gcn2 then phosphorylates the translation initiation factor 2a (eIF-2a), leading to reduced global protein synthesis and increased expression of amino acid biosynthetic enzymes (21). The signal transduction pathway governing Gcn2 is called general amino acid control. Like RelA, Gcn2 and Gcn1 bind to ribosomes, and previous findings support the idea that Gcn1 affects A-site function (53).

In S. cerevisiae there are four Obg/CgtA subfamily members that also appear to play roles in ribosome function: Nog1 (Ypl093w), Mtg2 (Yhr168w), Yal036c, and Ygr173w. Nog1 is a nuclear protein that plays a key role in assembly of the large ribosomal subunit; depletion of Nog1 leads to a decrease in 60S subunit assembly and formation of halfmer polysomes (12, 28, 31). Mtg2 associates with the large mitochondrial ribosomal subunit, is critical for mitochondrial translation, and is required for the maintenance of proper ribosomal subunit ratios (8).

The two remaining S. cerevisiae Obg/CgtA proteins, Yal036c and Ygr173w (hereafter called Rbg1 and Rbg2 for ribosome binding GTPase), belong to the DRG subgroup of Obg/CgtA proteins. Rbg1 and Rbg2 are tripartite proteins that are 52% similar to each other (Fig. 1) and ubiquitously found in all eukaryotes and archaebae sequenced to date. These proteins are similar to the other Obg/CgtA proteins only in the guanine nucleotide-binding domain (amino acids [aa] 69 to 275 and aa 67 to 275 for Rbg1 and Rbg2, respectively). Within this conserved GTPase domain, the Rbg proteins also have a 67-aa insertion of unknown function between the conserved G3 and G4 motifs (Fig. 1). This insertion sequence is unique to the

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eukaryotic and archaeal DRG subfamily (as determined by PSI BLAST searches).

The N and C termini of the Rbg proteins are distinct from those of the nucleolar, mitochondrial, and bacterial Obg/CgtA proteins. The N-terminal amino acids of the Rbg proteins (aa 1 to 68 and aa 1 to 66 for Rbg1 and Rbg2, respectively), predicted to contain two adjacent helices of unknown function, are strongly conserved (45% identical). The C terminus of the Rbg proteins (aa 290 to 368) contains a TGS domain (69), a universally conserved G1 through G4 motifs of the guanine nucleotide binding domain, which is indicated. An amino acid reference scale is provided.

Several lines of evidence suggest that the cytoplasmic Rbg proteins are involved in ribosome function. First, the expression pattern for RBG1 displays under various conditions clusters of genes involved in ribosome and rRNA biosynthesis (67). In addition, the RBG1 promoter has a conserved element found in many genes involved in ribosome function (67). Moreover, Rbg1, as well as other proteins involved in translation initiation, was copurified in a complex with eIF4G1-TAP (14, 15). The related gene, RBG2, is syntetically combined in a deletion of the large ribosomal subunit genes rpl22a and rpl6b (N. J. Krogan, unpublished data).

We show here that Rbg1 associates with polyribosomes but not with the 40S or 60S subunits or with 80S monosomes, indicating that Rbg1 specifically associates with translating ribosomes. Interacting partners of Rbg1 were identified by yeast two-hybrid. Among the interaction partners was a protein of unknown function, Gir2, which has sequence similarity to the N-terminal GI (Gcn2 and Impact) domain of Gcn2 that is involved in Gcn1 binding. We found that Gir2 also associates with ribosomes, and we have several lines of evidence showing that Gir2 binds to Gcn1 via its GI domain. Gir2 overexpression diminishes Gcn2 function, and this could be reverted by Gcn2 overexpression, suggesting that Gir2 competes with Gcn2 for Gcn1 binding. The polyribosome association of Gir2 was not dependent on Rbg1, although its association with polyosomes was in part dependent on Gcn1. Based on the connection between Rbg1 and Gir2 and between Gir2 and the components of the general amino acid control pathway, Rbg1 and Gir2 may play a role in adjusting the cell to stress conditions.

FIG. 1. Rbg1 and Rbg2 are tripartite proteins, here illustrated as a diagram. Shaded regions represent regions of high similarity. The universally conserved GI through G4 motifs of the guanine nucleotide binding domain are indicated by overlines. The C-terminal TGS domain is indicated. An amino acid reference scale is provided.

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**TABLE 1. Plasmids used in this study**

| Plasmid     | Description                                      | Source or reference |
|-------------|--------------------------------------------------|---------------------|
| pACT2      | GAL4 activation domain; LEU2                     | Clontech            |
| pAS2       | GAL4 binding domain; TRP1                       | Clontech            |
| pLAM5      | Human lamin C fused to the GAL4-BD              | Clontech            |
| pGAL1      | 2μ GAL1 URA3                                     | 45                  |
| pES125-9-1 | 2μ GAL1 GST URA3                                 | 53                  |
| pRS423     | 2μ HIS3                                         | 7                   |
| pJM1336    | pAS2-Rbg1                                       | This study          |
| pJM1617    | pAS2-GIR2                                       | This study          |
| pJM2648    | pAS2-GIR2N                                      | This study          |
| pJM4550    | pAS2-GIR2C                                      | This study          |
| pJM2651    | pAS2-GCN2(GI)                                   | This study          |
| pJM2646    | pACT2-GCN1(GI)                                  | This study          |
| pJM3958    | pES125-9-1-GIR2                                 | This study          |
| pJM2653    | p426GAL1-GIR2RN                                 | This study          |
| pJM4563    | p426GAL1-GIR2C                                  | This study          |
| pJM2621    | p426GAL1-GIR2                                   | This study          |
| pJM2749    | p426GAL1-GCN2(GI)                               | This study          |
| pAH15      | Ycp13-GCN2                                      | 21                  |
| p180       | GCN4-ace2 reporter plasmid                      | 44                  |

**MATERIALS AND METHODS**

**Construction of plasmids.** Plasmids used for the present study are listed in Table 1, and the oligonucleotides used in constructing plasmids for the present study are presented in Table 2. pJM1336, a pAS2 bait vector expressing a translational fusion between the GAL4 DNA-binding domain (GAL4-BD) and Rbg1, was constructed as follows. The Rbg1 gene was PCR amplified from yeast genomic DNA with primers Fun11*NcoI and 11up. The product was digested with NcoI/SalI and ligated into similarly digested pET28a (Novagen) to create pJM957. An NcoI/SalI-digested pAS2 to create pJM1336. Sequences encoding full-length Rbg1 and Gir2 may play a role in adjusting the cell to stress conditions.

**TABLE 2. Oligonucleotides used in this study**

| Primer                     | Sequence (5’–3’) |
|----------------------------|-----------------|
| Fun11*Nco                   | GTAAGCAGCTATCGCTAACA |
| 11up                       | CTATGATGTCGACCTT |
| Fun11PMI                    | CCGGATCTTACACCGCTTTGTTGTT |
| 173*Nco                    | GCATACACCATCGGTATATT |
| 173down                    | CTTGAGAAGACGTTTC |
| YDR152R1                   | ATGACATAGGGATTCTTAAAT |
| TAAGGAGAAGATGAGTAAAT |
| YDR152F2                   | GACGTAGGCGACCGTCCACGAG |
| YDR152Up                   | CCGAATCCCGAATCCGAGTAC |
| YDR152n                   | CCGCTCGTTATAGGTTAGGTT |
| Gir2c                      | CTATTGCAGGAGAGAGAGGAG |
| Fun11 KO start             | GAGCAATAGAGAGAGCTTCAGGTTGTT |
| Fun11 KO stop              | GTTGGAGCAAGAGATGAGTAC |
| YDR152Down                 | TTAGGATCCAGTCCAGTCGAG |
| GCN1(GCN2 BD) reverse       | CCGTGAAGGAGAGAGAGGAG |
| GCN2 FORWARD               | AAGAATAATATACG |
| GCN2 N                     | GCATTCATATCAGTCTTCTT |
| Gir2Xba1UP                 | ACTCTACATACATAGAGTATATAA |
| Gir2salDOWN                | TTAAGGAGCAAGAATATTTTATGAT |
TABLE 3. Strains examined in this study

| Strain | Relevant genotype | Source or reference |
|--------|-------------------|---------------------|
| Y190   | MATA gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL1-lacZ | 17 |
| Y187   | MATA gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL1-lacZ | 17 |
| YW5-1B | MATA trp1 ura3-52 leu2-3,112 | 63 |
| W303   | MATA trp1 ura3-52 leu2-3,112 | 62 |
| BY4704 | MATA ade2::HisG his3-200 leu2-300 met1020 trp1Delta3 ura30 | ATCC |
| BY4705 | MATA ade2::HisG his3-200 leu2-300 met1020 trp1Delta3 ura30 | ATCC |
| CRY1   | MATA ura3-52 trp1 ura3-52 his3-11 15/ his3-11,15 ura3/ura3 | 5 |
| H1511  | MATA ura3-52 trp1-63 leu2-3,112 GAL2 | 11 |
| H2557  | MATA ura3-52 trp1-63 leu2-3,112 gcn2Δ GAL2 | 11 |
| Y4741  | MATA his3 leu2 met15 ura3 | Yeast deletion collection |
| Y4742  | MATA his3 leu2-his3 ura3 | Yeast deletion collection |
| 370    | Same as Y4741 except rbg1::KanMX4 | Yeast deletion collection |
| 14803  | Same as Y4742 except ygr173p::KanMX4 | Yeast deletion collection |
| 14562  | Same as Y4742 except gcn1::KanMX4 | Yeast deletion collection |
| 1350   | Same as Y4742 except gir2::KanMX4 | Yeast deletion collection |
| JM2064 | rbg1::His3 rbg2::KanMX4 | This study |
| JM3185 | MATA Gir2::3HA::His3 | This study |
| JM3186 | MATA Gir2::3HA::His3 | This study |
| JM3370 | rbg1::KanMX4 Gir2::3HA::His3 | This study |
| JM3372 | gen1::KanMX4 Gir2::3HA::His3 | This study |

pAS2-GIR2 (pJM2648), pAS2-GIR2C (pJM5450), and pAS2-GCN2 (G1) (pJM2651). Sequences encoding the GI binding domain (aa 2047 to 2383) of GCN1 were amplified by PCR from yeast genomic DNA using the oligonucleotides GCN1 (GCN2 BD) forward and GCN1 (GCN2 BD) reverse and cloned into pACT2 using Neo and BamHI (restriction sites were designed in the oligos), resulting in pACT2-GCN1 (G1) (pJM2646).

The pAS2-GIR2, pAS2-GIR2N, pAS2-GIR2C, and pAS2-GCN2(G1) plasmids were digested with NheI and BamHI, and the fragments were ligated to SpeI/BamHI-digested p426GAL1 (45), placing the genes under the control of the galactose-inducible GAL1 promoter, thus creating p426GAL1-GIR2N (pJM2621), p426GAL1-GIR2C (p JM4563), and p426GAL1-GCN2(G1) (pJM2749).

Full-length GIR2 was cloned as a glutathione S-transferase (GST) fusion protein to construct pMI3958 as follows. GIR2 was PCR amplified from yeast genomic DNA by using the primers Gir2Xba1UP and Gir2Sal1DOWN and cloned as a Bsal/XbaI fragment into pESE128-9.1. The expression of these genes was verified by immunoblotting (data not shown).

Yeast strains. Yeast strains used for the present study are listed in Table 3. A Gir1 HA chromosomal fusion was generated as previously described (40). Briefly, the hemagglutinin (HA) tag, along with a selectable marker (His3MX6), was PCR amplified (using pFA6-3HA-His3MX6) by using the primers YDR152R1 and YDR152F2 (Table 2), and the PCR product was transformed into the yeast strains BY4704 and BY4705 to generate Gir1-HA (JM3185 and JM3370). JM3372 is a sporulation product from a diploid created by mating the gir1 deletion strain (i.e., strain 14562) with JM3186 (gcn1 Δ) (Table 2). The resulting supernatant was further clarified at 9,200 rpm for 10 min. Where indicated, the 9,200 rpm supernatant was incubated on ice for 30 min in the presence of 5 μg/ml of micrococcal nuclease and 3 mM CaCl₂, before loading onto sucrose gradients. We loaded 10 OD₂₆₀ units of cell extract in LBS and 1 mM dithiothreitol onto 10-ml 7 to 47% sucrose gradients that were centrifuged at 28,000 rpm for 4 h in an SW41 Ti rotor. Then, 50-μl fractions were collected manually using an ISCO UV monitor with a 254-nm filter. Samples were concentrated, and the sucrose was removed by the addition of water to 1 ml, followed by incubation on ice with 30 μl of 1% deoxycholate for 10 min. Next, 150 μl of 100% trichloroacetic acid was added, and the samples were incubated on ice overnight. Samples were centrifuged at 13,000 rpm for 10 min, vacuum dried, and resuspended in 1 M Tris and 8 mM sodium dodecyl sulfate (SDS) sample loading buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to nitrocellulose membrane. Immunoblot analysis was performed as described below.

Detection of protein-protein interaction by yeast two-hybrid analysis. pJM336 was transformed into the yeast strain Y190, and the resulting strain was transformed with a yeast genomic library cloned into pGAD-C1 (27). Approximately 200,000 transformants were selected on SD plates lacking histidine, leucine, and tryptophan (SD–His–Leu–Trp) supplemented with 60 mM 3-aminotriazole (3-AT). Each transformant was screened for lacZ activity by an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) colony filter lift (4). Transforms that resulted in expression (above background levels) from both the HIS3 and the lacZ reporter genes were further assayed for lack of reporter gene activation (i) in the absence of pJM1336, (ii) in the presence of pAS2 alone, and (iii) in the presence of pLAM5 (human lamin C protein fused to the GAL4 BD). Transformants that met all three criteria were mated to a pMI336 containing Y187 strain to reconfirm interaction, and their library clones were sequenced to identify the genes encoded.

All pAS2 clones were transformed into Y190, pACT2-GCN1 (G1) was transformed into Y187, the strains were mated, and the resulting diploids were assayed for HIS3 reporter gene activation by monitoring the growth of serial dilutions of liquid cultures on SD–His–Leu–Trp plates supplemented with 60 mM 3-AT. Expression of all fusion proteins was verified by immunoblot analysis as described below.

GST pulldown assays. To prepare extracts from yeast cells expressing GST (pES128-9.1) or GST-Gir2 (pJM3958) under a galactose-inducible promoter, cells were grown to mid log phase in SD lacking uracil (SD–Ura) with 4% raffinose, galactose was added to a final concentration of 2%, and the cultures were further incubated at 30°C for 4 h. Cells were harvested, washed once, and resuspended in HEPS buffer (50 mM HEPS [pH 7.6], 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 25 mM β-glycerophosphate, 0.1 mM Na₂VO₄, and 1 mM dithiothreitol containing the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 2 μg of leupeptin/ml, 2 μg of aprotinin/ml, 1.25 mM benzamidine, and 0.5 μg of pepstatin A/ml). Cells were lysed in the presence of glass beads,
FIG. 2. Rbg1 associates with polyribosomes. (A) Whole-cell extracts from strain W303 were resolved by density sedimentation in 7 to 47% sucrose gradients. (B) Polyribosomes were disrupted by the addition of micrococcal nuclease (5 U/µl) prior to loading of extracts on gradients. The UV absorbance trace (254 nm) obtained during fractionation is shown with the positions of the 40S, 60S, 80S, and polyribosomes indicated. Fractions (numbered) were analyzed by immunoblotting with antibodies against Rbg1, Rbg2 and S2, a small ribosomal subunit protein, as indicated. L, 1/100 cell extract loaded on gradient.

**RESULTS**

Rbg1 cofractionates with polyribosomes. All of the Obg/CgtA proteins examined thus far associate with the large ribosomal subunit (8, 12, 31, 39, 51, 59, 60, 70). To test whether this was also the case for Rbg1 and Rbg2, cell lysates were subjected to sucrose density gradient centrifugation and fractionated, and the cosedimentation of Rbg1 and Rbg2 with ribosomes was determined by immunoblotting. Antibodies to the small ribosomal subunit protein S2 were used as reference to monitor the migration and/or fractionation of the 40S ribosomal subunit, the 80S monosomes, and the polyribosomes. Rbg1 was found exclusively in the polyribosome fractions (Fig. 2A, fractions 11 to 20), a localization pattern distinctly different from that of the nucleolar, mitochondrial, and bacterial Obg/CgtA proteins. The polysome association detected here for Rbg1 would be consistent, however, with a cytoplasmic localization for the Rbg1 protein. The association between Rbg2 and ribosomes is unclear since Rbg2 fractionated throughout the gradient, with the majority of Rbg2 being in the lighter fractions. To confirm that the observed migration of Rbg1 was due to an association with polyribosomes and not due to association with another large protein complex, migration on sucrose gradients was examined after disruption of the polyribosomes with micrococcal nuclease. The addition of micrococcal nuclease to cell lysates results in cleavage of the mRNA between translating ribosomes which, in turn, results in a significant reduction in polyribosomes and a concomitant increase in 80S monosomes (Fig. 2B). Under these conditions, Rbg1 specifically cofractionated with the 80S monosomes (Fig. 2B, fractions 10 to 14). After mRNA digestion the distribution of Rbg2 in the polyribosome fractions, however, was un-
It is not known whether Rbg2 and Rbg1 are functionally redundant, since no phenotypes have been assigned to either the conditions assayed (Fig. 2).

**Identification of Rbg1 interacting partners by yeast two-hybrid screening.** In order to further investigate the cellular function of Rbg1, we attempted to identify potential interacting partners of Rbg1 through a yeast two-hybrid screen using Rbg1 fused to the GAL4-BD as the bait protein. Approxi-mately 200,000 Gal4-activating clones (GAL4-AD) were screened from a random library, and three distinct gene products—Tma46 (Yor091w), Ygr250c, and Gir2 (Ydr152w)—were identified as encoding Rbg1 interacting partners (Fig. 3). The smallest Rbg1-interaction partner of Gir2 was analyzed upon removal of ribosomes by cycloheximide and anisomycin was similar and only marginally superior for the interaction between Gir2 and Rbg1. To confirm the ability of Gir2 to interact with Rbg1 in vivo, we tested whether Gir2 also interacts with Gcn1 in vivo using GST-Gir2 mediated glutathione precipitation. We found that Gir2 interacts with Rbg1 and Gcn1. Of the proteins identified in the yeast two-hybrid screen, we focused on the interaction between Rbg1 and Gir2. To confirm the ability of Gir2 to interact with Rbg1, GST-tagged Gir2 was overexpressed in wild-type cells and precipitated using glutathione-Sepharose, and the precipitates were analyzed by immunoblotting. Rbg1 was easily detectable as a copurifying protein in the extracts containing GST-Gir2 but not with the GST-negative control (Fig. 4A). To determine whether an RNA species was necessary for the interaction between Gir2 and Rbg1, the pulldown analyses were repeated in the presence of RNase A. The interaction between Gir2 and Rbg1 was not altered upon digestion of RNA (Fig. 4A). In addition, the interaction between Gir2 and Rbg1 was analyzed upon removal of ribosomes by centrifugation of the samples at 100,000 × g. Under these conditions the interaction between Gir2 and Rbg1 persisted (data not shown). We conclude that Gir2 and Rbg1 interact in vivo.

The GI domain of Gcn2 is necessary and sufficient for in vivo binding to Gcn1, a key regulatory protein in general amino acid control pathway(34, 53). Since the N terminus of Gir2 shares sequence similarity with the GI domain of Gcn2 (34), we tested whether Gir2 also interacts with Gcn1 in vivo using GST-Gir2 mediated glutathione precipitation. We found that Gcn1 was specifically detected in the GST precipitation of cell
extracts harboring GST-Gir2 (Fig. 4A), suggesting that, in addition to Rbg1, Gir2 also interacts with Gcn1 in vivo. The interaction between Gir2-Gcn1 was stable in either the presence of RNase (Fig. 4A) or the absence of ribosomes (data not shown).

We next analyzed the interaction between Gir2 and Gcn1 by using the yeast two-hybrid analysis. The Gcn2 binding domain of Gcn1 (aa 2048 to 2383), called the GIB domain (for GI binding), has been previously defined (34, 53). As expected, therefore, cells expressing Gcn1(GIB)-AD and Gcn2(GI)-BD on the appropriate selective medium grew, whereas cells harboring Gcn1(GIB)-AD and the vector control pAS2 did not (Fig. 4B). Consistent with the ability of GST-Gir2 to precipitate Gcn1, we observed growth for cells expressing Gcn1(GIB)-AD and Gir2-BD on the appropriate selective medium. We conclude that full-length Gir2 interacts with Gcn1.

To determine whether the N-terminal GI domain of Gir2 was sufficient for its interaction with Gcn1(GIB)-AD, the N terminus (aa 1 to 170) or C terminus (aa 167 to 265) of Gir2 was expressed as a fusion protein with the GAL4 DNA-binding domain (Gir2N-BD and Gir2C-BD, respectively). Expression of these truncated fusions was verified by immunoblotting (data not shown). Cells expressing Gcn1(GIB)-AD and Gir2N-BD showed growth comparable to that of the positive control, whereas cells expressing Gcn1(GIB)-AD and Gir2C-BD did not grow, suggesting that the GI domain of Gir2 is necessary and sufficient for its interaction with Gcn1 (Fig. 4B).

To provide evidence that Gir2 and Gcn1 interact in vivo, we exploited the well-characterized general amino acid control pathway. This signal transduction pathway enables cells to overcome amino acid starvation. In particular, direct physical interaction between Gcn1 and Gir2 is essential for activating Gcn2 and thus the general amino acid control (21, 53). The function of this pathway can be assayed by growing cells on medium containing 3-AT, an inhibitor of the histidine biosynthetic enzyme encoded by HJS3. Overproduction of the GI domain of Gcn2 results in a dominant-negative phenotype, observable as the lack of growth on 3-AT containing medium (13, 34; Fig. 5A). The lack of growth is due to the GI domain competing with native Gcn2 for Gcn1 binding, thereby dampening activation of the general amino acid control.
ening Gcn2 activation and consequently diminishing the ability of cells to overcome amino acid starvation imposed by 3-AT (53). Simultaneous coexpression of Gcn2 overcomes the dominant-negative phenotype (Fig. 5A) (53). We posited that if Gir2 binds to the same domain in Gcn1 as Gcn2 does, then overexpression of Gir2 would also titrate Gcn1 and lead to a dominant-negative phenotype. To test this, we induced expression of full-length Gir2 (under the control of the galactose inducible GAL1 promoter) in an otherwise wild-type strain. We observed a severe growth inhibition on medium containing 3-AT (Fig. 5A), while co-overexpression of Gcn2 restored growth. Furthermore, suppression of the 3-AT sensitivity phenotype by Gcn2 was not simply due to a growth advantage conferred to cells overexpressing Gcn2, since wild-type cells overexpressing Gcn2 grew less well than cells harboring a vector alone under starved conditions (Fig. 5B). Taken together, these data support the model that overexpressed Gir2 competes with Gcn2 for Gcn1 binding by utilizing the same or overlapping binding domains in Gcn1 and thereby dampens signaling of Gcn1 to Gcn2 under amino acid limiting conditions.

We next sought to determine whether the GI domain of Gir2 alone was sufficient to produce the dominant-negative phenotype. Overexpression of Gir2N in the presence of 3-AT resulted in a dominant-negative phenotype, whereas overexpression of Gir2C had no effect on cell growth (Fig. 5A). Thus, we conclude that the N-terminal GI domain of Gir2 is both necessary and sufficient for binding Gcn1 in vivo.

The basal Gcn2 enzyme activity, as well as the activation of Gcn2 under amino acid starvation, is dependent on direct Gcn1-Gcn2 interaction. If the dominant-negative phenotype of Gir2 overexpression is due to Gir2 disrupting Gcn1-Gcn2 interaction, then this should result in reduced phosphorylation of the Gcn2 substrate, eIF-2α, even under amino acid-replete conditions. To test our prediction, we examined the phosphorylation state of eIF-2α in strains overexpressing GST-Gir2. As expected, the levels of eIF-2α phosphorylation were dramatically decreased in comparison to cells overexpressing GST alone (Fig. 5C). These data further support the conclusion that Gir2 binds Gcn1 and, at least when Gir2 is overexpressed, competes with Gcn2 for Gcn1 binding and thereby inhibits the activation of Gcn2 by Gcn1.

Although our results support the idea that Gir2 is a binding partner for Gcn1, it is not clear that the main role of Gir2 is in regulating the general amino acid control pathway. Therefore, we examined whether the gir2Δ mutant had an effect on the output of a Gcn2-regulated gene, GCN4. Isogenic wild-type, yih1Δ, gir2Δ, and gcn2Δ strains were transformed with a plasmid (p180) harboring a GCN4-lacZ reporter construct in which the translation of β-galactosidase is under the control of the GCN4 5′ untranslated region containing four upstream open reading frames, and β-galactosidase activity was assayed under nonstarvation and starvation conditions. In contrast to a Gcn2 deletion, a GIR2 deletion did not alter the level of GCN4 expression (Fig. 5D). Thus, in vivo, it is unlikely that Gir2 plays a general role in controlling Gcn2 activity. Similarly, deletion of another gene, YIH1, also encoding a GI domain known to bind Gcn1 (54), also resulted in no increase in GCN4 expression (Fig. 5D), in agreement with previous findings that deletion of YIH1 did not lead to increased eIF-2α phosphorylation (54).

Gir2 comigrates with ribosomes. Thus far, we have shown that Gir2 interacts with Rbg1 (Fig. 3 and 4) and Gcn1 (Fig. 4). Moreover, both Rbg1 and Gcn1 are associated with polyribosomes (Fig. 2; 41). Therefore, we sought to determine whether Gir2 was also ribosome associated. We epitope tagged the chromosomal GIR2 gene and subjected the resulting strain to velocity sedimentation through a sucrose gradient to resolve ribosomes. Under the conditions assayed, the vast majority of Gir2-3HA migrated at the top of the sucrose gradient (Fig. 6A), indicating that the majority of Gir2-3HA is either not associated with larger complexes or becomes dissociated from larger complexes during centrifugation. A significant amount of Gir2-3HA, however, was found in fractions corresponding to polyribosomes (Fig. 6A). Interestingly, this pattern of fractionation is similar to that previously reported for Gcn1 (42). Furthermore, the Gir2-3HA levels appear to increase with the size of the polyribosomes, a profile similar to that seen for the small ribosomal subunit protein S2 (Fig. 6A). Upon disruption of the elongating ribosomes by the addition of micrococcal nuclease, the amount of Gir2 found in the polyribosome fractions decreased, and there was an increase in the signal corresponding to the monosome peak (Fig. 6B). Thus, at least a proportion of the population of Gir2 is associated with the polyribosomes.

The association of Gir2 with polyribosomes is partially dependent on Gcn1. Gir2, Rbg1, and Gcn1 each associate with polyribosomes (Fig. 2 and 6; 42), and Gir2 interacts with both Rbg1 and Gcn1 (Fig. 3 and 4). It is therefore possible that, on the translating ribosome, Gir2 interacts with both Rbg1 and Gcn1 simultaneously, with its N-terminal GI domain tethered to Gcn1 and its C terminus tethered to Rbg1. To determine whether the association of Gir2 with the polyribosomes was a direct interaction or was dependent on either of its binding partners, we examined the fractionation pattern of Gir2-3HA on sucrose density gradients in strains lacking either RBG1 or GCN1.

In the absence of Rbg1, the distribution of Gir2-3HA in
Thus, the polysome association of Rbg1 in cells lacking Gir2 is indistinguishable from that of wild type, independent of Gcn1. Likewise, the fractionation pattern of Rbg1 in cells lacking Gcn1 was identical to that of wild type (Fig. 7C compared to Fig. 7A). Furthermore, the polysome association of Rbg1 can be restored by expression of Gcn1 from a plasmid (data not shown). Thus, the polysome association of Gir2 is at least partially dependent on Gcn1 but not on Rbg1.

We also examined the requirements of Rbg1 for polysome association. The Rbg1 fractionation pattern in cells lacking Gcn1 was identical to that of wild type (Fig. 7C compared to Fig. 7A), indicating that the ribosome association of Rbg1 is independent of Gcn1. Likewise, the fractionation pattern of Rbg1 in cells lacking Gir2 is indistinguishable from that of wild type (data not shown). Thus, the polysome association of Rbg1 is independent of Gir2 and Gcn1.

DISCUSSION

Here we show that Rbg1, a member of the Obg/CgtA subfamily of GTPases, is associated with polyribosomes. This finding is in agreement with the prediction that all Obg/CgtA proteins are involved in ribosome function (6, 38). Unlike the nucleolar, mitochondrial, and bacterial Obg/CgtA proteins examined that specifically interact with the large ribosomal subunit, however, Rbg1 cofractionates with polyribosomes (Fig. 2). Thus, it is likely that Rbg1 plays a cellular role distinct from the large subunit assembly role filled by the Nog1 and Mtg2 proteins (8, 12, 28, 31).

The nature of the polysome association of Rbg1 is unclear. It is possible that a specific translational factor(s) recruits Rbg1 to the ribosome only after initiation of translation or that Rbg1 binds to a specific ribosome conformational state that only occurs during translation elongation. Alternatively, Rbg1 could interact directly with tRNA and remain associated with the translating ribosome due to a dynamic tRNA association. Finally, it is possible that Rbg1 associates with a specific subset of polysomes, such as the subpopulation of ribosomes that are stalled because the required aminoacyl-tRNA is not available or because the ribosome has accepted an uncharged tRNA rather than a charged one. This latter possibility is particularly intriguing given that uncharged tRNAs can activate both the TGS-containing RelA protein in bacteria and the Gcn1 interacting partner, Gcn2, in yeast (21, 57).

We identified potential interacting partners of Rbg1: Yap1, Ygr250c, Tma46, and Gir2. We focused here on the interactions between Rbg1 and Gir2, Gir2 and Gcn1, and all three of these proteins with polyribosomes. We show that Gir2 interacts with Rbg1 and Gcn1 in physically distinct areas with the Gir2 C terminus binding to Rbg1 (Fig. 3) and the N-terminal GI domain binding Gcn1 (Fig. 5). Thus, Gir2 could interact with both Rbg1 and Gcn1 simultaneously. Moreover, we show that in ribosome cosedimentation assays, Gir2 bound to ribosomes in a manner that is at least in part dependent on Gcn1, whereas the Gir2-ribosome interaction was independent of Rbg1 and the Rbg1-ribosome interaction was independent of Gcn1. We therefore propose that Gir2 acts as a linker between Rbg1 and Gcn1, perhaps to mediate signals from Gcn1 to Rbg1. One attractive possibility is that hydrolysis of GTP by Rbg1 could be the output of this signaling cascade (Fig. 8B).

We propose that Gcn1, Gir2, and Rbg1 may represent another polysome-associated, response system (Fig. 8B). One exciting possibility is that, in the course of evolution, there was a shuffling of protein domains between the two interacting bacterial proteins (Obg/CgtA and SpoT) that resulted in the archaeal and eukaryotic hybrid Rbg1-like protein consisting of the GTP-binding domain of Obg/CgtA and the TGS domain of SpoT. Although mechanistically different, it has been postulated that the RelA-mediated response to stress (i.e., amino acids) is modeled by the interaction between translating ribosomes and Gcn2, Gcn1, Rbg1, and Gir2. (A) Gcn1 interacts with Gcn2 on the ribosome for detecting uncharged tRNAs under amino acid starvation. This leads to the stimulation of the Gcn2 kinase domain, subsequent phosphorylation of its substrate eIF-2α, and finally to the activation of the general amino acid control pathway described in panel A, the Rbg1 pathway may receive a signal from Gcn1 that is relayed through Gir2. It is possible that this signal alters the guanine nucleotide bound state of Rbg1 to elicit an as-yet-undefined biological response.
acid starvation) is analogous to the Gcn1/Gcn2 response in *S. cerevisiae* (42, 53). RelA, a polyosome-associated protein, is activated by the binding of uncharged tRNAs (46). The SpoT protein is 50% similar to RelA (19). E. coli CgtA, interacts with and controls the activity of SpoT, on the ribosome (30, 70), clearly implicating this bacterial GTPase in stress response. In *S. cerevisiae*, during amino acid starvation, induction of the Gcn2 kinase activity requires association of Gcn2 with uncharged tRNA and Gcn1, which is proposed to be directly involved in relaying this signal to Gcn2 (Fig. 8A; 21). Gcn2, contains a domain similar to that of histidyl-tRNA synthetases that is important for binding to uncharged tRNA (68). Conceivably, the TGS domain of Rbg1 could also bind uncharged tRNA, specifically threonyl-tRNA, on a subset of ribosomes. In a manner analogous to the activation of Gcn2, Rbg1 binding to uncharged tRNA, coupled with a specific signal (e.g., an as-yet-unknown stress condition) relayed by Gcn1 through Gir2, could result in an as-yet-undefined Rbg1-mediated biological response (Fig. 8).

The high sequence conservation of Rbg1 and Rbg2 orthologs suggests that these proteins are involved in fundamental pathways and perhaps their binding partners are also conserved. In fact, the mammalian Rbg proteins interact with protein orthologs we identified in the present study, with some interesting differences: mammalian DRG1 (ortholog to yeast Rbg1) binds to DFRP1 (Tma46), and DRG2 (Rbg2) binds to DFRP2 (Gir2) (24). These DRG proteins are susceptible to degradation through the ubiquitin-mediated pathway, and their degradation is prevented by their interaction with DFRP proteins. Thus, the DFRP proteins provide a means of regulating DRG protein levels, which may be important for their function in tissue specific and developmentally specific expression (56). DFRP1 and DFRP2 share sequence similarity in a small region that is involved in DRG binding, but otherwise they are very different in domain structure. The Tma46 and Gir2 fragments we identified as being sufficient for a two-hybrid interaction with Rbg1 coincided largely with the DRG binding sites of the mammalian proteins, a finding consistent with the idea that their functions are evolutionary conserved.

If Gir2 can compete with Gcn2 for the binding of Gcn1 in vivo, Gir2 could be expected to act as a negative regulator of Gcn1, maintaining Gcn1 in an inactive state until an environmental stress signal is present. This appears not to be the case, however, since deletion of *GIR2* did not lead to increased derepression of GCN4 as determined by *GCN4-lacZ* expression studies (Fig. 5D). On the contrary, it appears that Gir2 may have a slight positive regulatory role in Gcn2 activation, but only when cells are starved. Although further verification of such an additional positive regulatory role would be necessary, our findings suggest that, as proposed for Yih1 (54), Gir2 may only inhibit Gcn2 activation under specific circumstances or in specific cellular compartments when or where Gcn2 activation is disadvantageous to the cell.

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