

## *Saccharomyces cerevisiae* Rbg1 Protein and Its Binding Partner Gir2 Interact on Polyribosomes with Gcn1<sup>∇</sup>

P. K. Wout,<sup>1</sup>† E. Sattlegger,<sup>2</sup> S. M. Sullivan,<sup>1</sup> and J. R. Maddock<sup>1\*</sup>

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048,<sup>1</sup> and Institute of Molecular BioSciences, Massey University, Private Bag 102 904 NSMC, Auckland 0632, New Zealand<sup>2</sup>

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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 GI (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 GI 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 tRNA and relays the A-site occupancy information to the protein kinase Gcn2. Gcn2 then phosphorylates the translation initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ), 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 nucleolar 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 archaea 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

\* Corresponding author. Mailing address: Department of Molecular, Cellular, and Developmental Biology, University of Michigan, 830 North University, Ann Arbor, MI 48109-1048. Phone: (734) 936-8068. Fax: (734) 647-0884. E-mail: maddock@umich.edu.

† Present address: Department of Biological Sciences, 617 Fairchild Center, Columbia University, New York, NY 10027.

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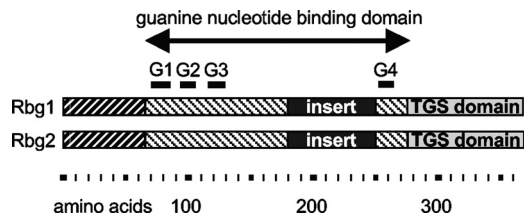


FIG. 1. Rbg1 and Rbg2 are tripartite proteins, here illustrated as a diagram. Shaded regions represent regions of high similarity. The universally conserved G1 through G4 motifs of the guanine nucleotide binding domain are indicated by overlines. The C-terminal TGS domain is indicated. An amino acid reference scale is provided.

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 sequence of ~50 aa that forms a  $\beta$ -sheet structure (32; Fig. 1). The function of the TGS domain is currently unknown, although a regulatory role has been suggested (3, 69). TGS domains are found in a limited number of proteins including threonyl-tRNA synthetases, DRG-like GTPases and, interestingly, in the (p)ppGpp synthetases SpoT and RelA.

Several lines of evidence suggest that the cytoplasmic Rbg proteins are involved in ribosome function. First, the expression pattern *RBG1* displays under various conditions clusters with 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 synthetically sick in combination with a deletion of the large ribosomal subunit genes *rpl22a* or *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 using a yeast two-hybrid screen. 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 polysomes 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.

TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pACT2	GAL4 activation domain; <i>LEU2</i>	Clontech
pAS2	GAL4 binding domain; <i>TRP1</i>	Clontech
pLAM5'	Human lamin C fused to the GAL4-BD	Clontech
p426GAL1	2 $\mu$ GAL1 <i>URA3</i>	45
pES128-9-1	2 $\mu$ GAL1 GST <i>URA3</i>	53
pRS423	2 $\mu$ <i>HIS3</i>	7
pJM1336	pAS2- <i>RBG1</i>	This study
pJM1617	pAS2- <i>GIR2</i>	This study
pJM2648	pAS2- <i>GIR2N</i>	This study
pJM4550	pAS2- <i>GIR2C</i>	This study
pJM2651	pAS2- <i>GCN2(GI)</i>	This study
pJM2646	pACT2- <i>GCN1(GIB)</i>	This study
pJM3958	pES128-9-1- <i>GIR2</i>	This study
pJM2653	p426GAL1- <i>GIR2N</i>	This study
pJM4563	p426GAL1- <i>GIR2C</i>	This study
pJM2621	p426GAL1- <i>GIR2</i>	This study
pJM2749	p426GAL1- <i>GCN2(GI)</i>	This study
pAH15	Yep13- <i>GCN2</i>	21
p180	<i>GCN4-lacZ</i> 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/SacI* and ligated into similarly digested pET28a (Novagen) to create pJM957. An *NcoI/SalI* *RBG1* fragment from pJM957 was then ligated to *NcoI/SalI*-digested pAS2 to create pJM1336. Sequences encoding full-length *GIR2*, its N terminus (aa 1 to 170) or C terminus (aa 167 to 265), or the N-terminal GI domain of *GCN2* (aa 1 to 125) were amplified by PCR from yeast genomic DNA by using the oligonucleotides YDR152Up/YDR152Down, YDR152Up/YDR152wN, *GIR2C*/YDR152Down, or *GCN2* FORWARD/*GCN2* N, respectively. These PCR fragments were cloned into pAS2 by using *NdeI* and *BamHI* (restriction sites were designed in the oligonucleotides), creating translational fusions with the GAL4-BD; the resulting plasmids were pAS2-*GIR2* (pJM1617),

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5'-3')
Fun11* <i>NcoI</i> .....	.....GATTGAGCACCATGGCTACTACA
11up .....	.....CCTATGATTGCGCACTT
Fun11PM1 .....	.....CGGGATCCTTAGCCCTTTGGTTAGT
173* <i>Nco</i> .....	.....GCACTACACCATGGGTATTATCG
173down .....	.....CGTTAGAGTAGGTTAGC
YDR152R1 .....	.....ATGCAGAAGAAAATACTAAATACT TAAGTACACAAGTATGAATTCGAG CTCGTTTAAAC
YDR152F2 .....	.....GACGTAGCCAAGGCACTTGCCAAGA CCGAAATAGCAAATCAACGGATCC CCGGGTTAATTAA
YDR152Up .....	.....GGATCATATGGATTATAAGGAAGA
YDR152wN .....	.....GGATCCTTACTGCTTCTCGAGC
<i>GIR2C</i> .....	.....CATATGCTCGAGAAGCAGTAC
Fun11 KO start .....	.....GCAGTAGGTGCAAGCTGAGAGTTGT TGATTGAGCAAATGAGATTGTAC TGAGTGCAC
Fun11 KO stop .....	.....GTTCCGAGAATCACTTTTCAAGATG GTAACAACATCTTCGCTGTGCGGT ATTCACAGCG
YDR152Down .....	.....TAGGATCCATGCAGAAGAAAATA
<i>GCN1(GCN2 BD)</i> forward .....	.....CCATGCCGGAAGTTGCTGGCTC
<i>GCN1(GCN2 BD)</i> reverse .....	.....GGATCCCTACATTTCTCTCTCA
<i>GCN2</i> FORWARD .....	.....AAGAATATATACTCC
<i>GCN2</i> N .....	.....GGATCCTTACAGTTTCTTCTTGA
<i>Gir2XbaI</i> UP .....	.....ACTTCTAGATACACTATGGATTATAA
<i>Gir2SalI</i> DOWN .....	.....TTAAGTCGACAAGTATTTATTGAT

TABLE 3. Strains examined in this study

Strain	Relevant genotype	Source or reference
Y190	<i>MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL1-lacZ</i> <i>LYS2::GAL1-HIS3 cyh<sup>R</sup></i>	17
Y187	<i>MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL1-lacZ</i>	17
YW5-1B	<i>MATa trp1 ura3-52 leu2-3,112</i>	63
W303	<i>MATa/MATα trp1/trp1 leu2-3 112/leu2-3 112 his3-11 15/his3-11,15 ura3/ura3</i> <i>can1-100/can1-100 ade2-1/ade2-1</i>	62
BY4704	<i>MATa ade2Δ::hisG his3Δ200 leu2Δ0 met10Δ0 trp1Δ63 ura3Δ0</i>	ATCC
BY4705	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 met10Δ0 trp1Δ63 ura3Δ0</i>	ATCC
CRY1	<i>MATa ura3-52 trp1 leu2-3,112 his3-11 ade2-1 can1-100</i>	5
H1511	<i>MATα ura3-52 trp1-63 leu2-3,112 GAL2<sup>+</sup></i>	11
H2557	<i>MATα ura3-52 trp1-63 leu2-3,112 gcn2Δ GAL2<sup>+</sup></i>	11
BY4741	<i>MATa his3 leu2 met15 ura3</i>	Yeast deletion collection
BY4742	<i>MATα his3 leu2 lys2 ura3</i>	Yeast deletion collection
370	Same as BY4741 except <i>rbg1::KanMX4</i>	Yeast deletion collection
14803	Same as BY4742 except <i>ygr173w::KanMX4</i>	Yeast deletion collection
14562	Same as BY4742 except <i>gcn1::KanMX4</i>	Yeast deletion collection
1350	Same as BY4742 except <i>gir2::KanMX4</i>	Yeast deletion collection
JM2064	<i>rbg1::HIS3 rbg2::KanMX4</i>	This study
JM3185	<i>MATa GIR2::3HA::HIS3</i>	This study
JM3186	<i>MATα GIR2::3HA::HIS3</i>	This study
JM3370	<i>rbg1::KanMX4 GIR2::3HA::HIS3</i>	This study
JM3372	<i>gcn1::KanMX4 GIR2::3HA::HIS3</i>	This study

pAS2-GIR2N (pJM2648), pAS2-GIR2C (pJM4550), and pAS2-GCN2 (GI) (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 NcoI and BamHI (restriction sites were designed in the oligos), resulting in pACT2-GCN1(GIB) (pJM2646).

The pAS2-GIR2, pAS2-GIR2N, pAS2-GIR2C, and pAS2-GCN2(GI) 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-GIR2 (pJM2621), p426GAL1-GIR2N (pJM2653), p426GAL1-GIR2C (pJM4563), and p426GAL1-GCN2(GI) (pJM2749).

Full-length GIR2 was cloned as a glutathione S-transferase (GST) fusion protein to construct pJM3958 as follows. *GIR2* was PCR amplified from yeast genomic DNA by using the primers Gir2Xba1UP and Gir2Sal1DOWN and cloned as a XbaI/SalI fragment into pES128-9-1. 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 Gir2 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 Gir2-HA (JM3185 and JM3186). JM3370 is a sporulation product from a diploid created by mating the *rbg1* deletion strain (370) with JM3186 (*GIR2::3HA::HIS3*). JM3372 is a sporulation product from a mating between the *gcn1* deletion strain (i.e., strain 14562) with JM3185 (*GIR2::3HA::HIS3*) and sporulating the resulting diploid to obtain haploid cells. The *rbg1 rbg2* mutant contains the *rbg2::KanMX4* allele from strain 14803 and an *rbg1::HIS3* mutant generated by integration of a PCR fragment generated by amplifying *HIS3* from pRS423 with oligonucleotides Fun11 KO start and Fun11 KO stop.

**Yeast polysome analysis.** Cells grown in 200 ml of yeast extract-peptone-adenine-dextrose (YPAD) medium at 30°C to an optical density at 600 nm ( $OD_{600}$ ) of 0.5 to 1.0 were transferred to a 250-ml centrifuge tube, to which 1 ml of a 10-mg/ml concentration of cycloheximide and ice (to fill the tube) were added. Cells were incubated on ice with periodic swirling for 10 min, harvested at 7,000 rpm for 5 min in an SLA 1500 rotor, and washed with 20 ml of ice-cold lysis buffer (LBS; 40 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2 mM  $MgCl_2$ , 50  $\mu$ g of cycloheximide/ml). The cell pellet was resuspended in 2.5 ml of LBS and transferred to a 15-ml Corex tube. Then, 1 ml of glass beads was added, and the cell suspension was vortexed six times for 30 s with 30-s rests on ice between bursts. An additional 2.5 ml of LBS was added, and the cell suspension was centrifuged at 6,500 rpm in a SA600 rotor to remove any unbroken cells. 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 U/ $\mu$ l of micrococcal nuclease and 3 mM  $CaCl_2$  before loading onto sucrose gradients. We loaded 10  $OD_{260}$  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, 500- $\mu$ l 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  $\mu$ l of 1% deoxycholate for 10 min. Next, 150  $\mu$ l 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 6 $\times$  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.** pJM1336 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- $\beta$ -D-galactopyranoside) colony filter lift (4). Transformants 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 pLAMS' (human lamin C protein fused to the GAL4-BD). Transformants that met all three criteria were mated to a pJM1336 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(GIB) 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 HEPES buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 10 mM  $MgCl_2$ , 1 mM EDTA, 25 mM  $\beta$ -glycerophosphate, 0.1 mM  $Na_3VO_4$ , and 1 mM dithiothreitol containing the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g of leupeptatin/ml, 2  $\mu$ g of aprotinin/ml, 1.25 mM benzamidine, and 0.5  $\mu$ g of pepstatin A/ml). Cells were lysed in the presence of glass beads,

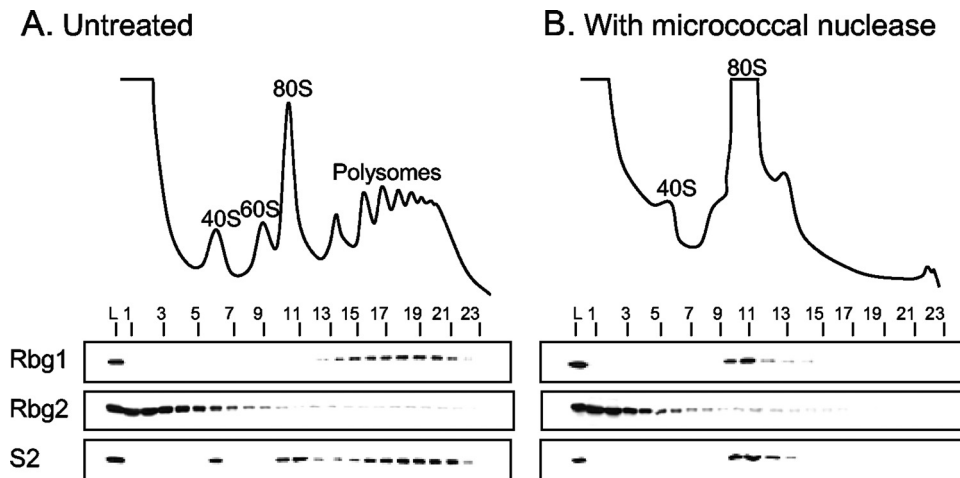


FIG. 2. Rbg1 associates with polyribosomes. (A) Whole-cell extracts from strain W303 were resolved by density sedimentation in 7 to 47% sucrose gradients. (B) Polyribosomes were disrupted by the addition of micrococcal nuclease ( $5 \text{ U}/\mu\text{l}$ ) prior to loading of extracts on gradients. 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.

and Triton X-100 was added to a final concentration of 1%. The lysates were centrifuged at  $20,800 \times g$  for 15 min to remove cell debris and, if ribosomes were to be removed, the resulting supernatants were further clarified by centrifugation at  $100,000 \times g$  for 10 min. Then,  $125 \mu\text{g}$  of RNase A was added for 30 min at  $4^\circ\text{C}$  to samples in which RNA species were digested. A total of 2 mg of cell lysate was mixed with  $20 \mu\text{l}$  of a 75% slurry of glutathione-Sepharose beads, followed by incubation at  $4^\circ\text{C}$  for 2 h with constant agitation. The glutathione beads were collected and then washed twice with HEPES buffer plus 1% Triton X-100, and bound proteins were eluted by boiling for 5 min in the presence of Laemmli SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE, and proteins were detected by immunoblotting as described below.

**Assaying  $\beta$ -galactosidase reporter gene activity.** Yeast strains were transformed with plasmid p180 (44) harboring the *GCN4-lacZ* reporter gene. For each plasmid, two independent transformants were grown to saturation and diluted to an  $\text{OD}_{600}$  of  $\sim 0.2$  in two identical cultures. After 2 h of growth, one set of cultures was supplemented with  $0.5 \mu\text{g}$  of Sulfometuron/ml to induce starvation for branched amino acids. Unstarved cultures were grown for a total of 6 h, and starved cultures were grown in the presence of Sulfometuron for 6 h. Enzyme assays were performed as previously described (43).

**Overexpression of GIR2 under derepressing conditions.** p426GAL1-GIR2 (pJM2621), p426GAL1-GIR2N (pJM2653), p426GAL1-GIR2C (pJM4563), and p426GAL1-GCN2(GI) (pJM2749), as well as the p426GAL1 vector itself, were transformed into yeast strain YW5-1B or YW5-1B containing pAH15 for expression of Gcn2 (22). Transformants were grown in SD-Ura-His liquid medium at  $30^\circ\text{C}$  to an  $\text{OD}_{600}$  of 1. Serial dilutions of the cultures were spotted onto SD-Ura or SD-Ura-His plates supplemented with 10 mM 3-AT in the presence of 2% glucose or 10% galactose. The plates were incubated at  $30^\circ\text{C}$  for 2 to 5 days.

**Analysis of in vivo eIF-2 $\alpha$  phosphorylation levels in cells overexpressing Gir2.** Yeast strains (H1511) expressing GST (pES128-9-1) or GST-Gir2 (pJM3958) and isogenic *gcn2 $\Delta$*  strain H2556 expressing GST (pES128-9-2) were grown to exponential phase in medium containing 2% galactose, subjected to starvation, and analyzed for eIF-2 $\alpha$  phosphorylation as described previously (52).

**Immunoblot analysis.** Rbg1 antibody (Sigma Genosys) was raised in rabbits against a truncated Rbg1 lacking the putative amphipathic helices (aa 1 to 302). Rbg1 was detected by using purified antibody, which had been treated with acetone powders derived from ATCC 4000370 at a dilution of 1:250 or 1:500. Antibody against Rbg2 (Sigma Genosys) was raised in rabbits against full-length Rbg2, affinity purified, and used at a dilution of 1:500. The specificity of these antibodies was verified in Western blots using wild-type and *RBG1* and *RBG2* deletion strains (data not shown). Gir2-HA fusions were detected using monoclonal anti-HA antibody (Covance) at a 1:1,000 dilution. The S2 antibody was a gift from the laboratory of Jonathon R. Warner and was used at a 1:2,000 dilution (66). Antibodies to eIF-2 $\alpha$  and Gcn1 were a gift from the laboratory of Alan G. Hinnebusch and were used at dilutions of 1:2,000 (9) or 1:1,000 (65),

respectively. eIF-2 $\alpha$  phosphorylated on Ser-51 was detected by using antibodies from BioSource International, Inc., at 1:5,000. All antibody reactions were performed in the presence of 5% nonfat dried milk as a blocking agent. Immunoblots were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences) as directed by the manufacturer.

## 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-

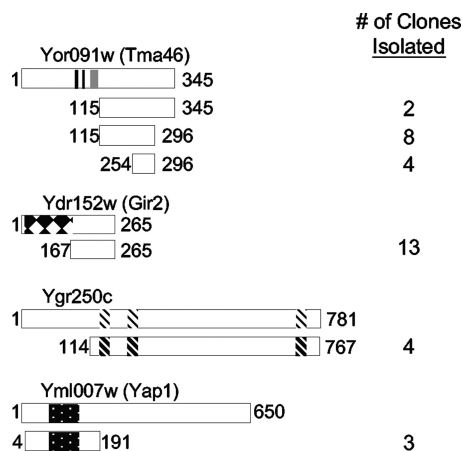


FIG. 3. Interacting partners of Rbg1. A yeast two-hybrid screen identified Tma46, Gir2, Ygr250c, and Yap1 as potential interacting partners of Rbg1. The encoded protein fragments are depicted under a diagram of each full-length protein. Beginning and ending amino acids are indicated, as well as the number of clones for each fragment. Tma46 encodes a potential nuclear localization sequence and a zinc finger domain as indicated by the black and gray bars, respectively. Gir2 contains an N-terminal GI domain (black and white diamonds), as discussed in the text. Ygr250c possesses three potential RNA-binding motifs (diagonals), where indicated. The well-characterized Yap1 contains a basic-leucine zipper domain (black with dots).

changed. We conclude that Rbg1 interacts specifically with translating ribosomes and that this interaction is stable after digestion of the intervening mRNA. Rbg2, in contrast, does not appear to be associated specifically with ribosomes under the conditions assayed (Fig. 2).

It is not known whether Rbg2 and Rbg1 are functionally redundant, since no phenotypes have been assigned to either mutant (*Saccharomyces* Genome Database and data not shown). Furthermore, we did not observe any obvious phenotypes when working with either the single or double deletion mutants (e.g., growth rate), suggesting that the protein synthesis rate is not severely affected. When scoring for sensitivity to some antibiotics we found that hygromycin B sensitivity was unchanged in *rbg1Δ*, *rbg2Δ*, and *rbg1Δrbg2Δ* strains. The sensitivity among these single- and double-deletion strains toward cycloheximide and anisomycin was similar and only marginally higher than that of wild type (data not shown). This finding may support the idea that the corresponding proteins have a function on the ribosome.

**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. Approximately 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 a minimum of four times each as interacting partners of Rbg1 (Fig. 3). Furthermore, Yap1 (Yml007w) was identified three times. Yap1 is a basic leucine zipper transcription factor that is essential for the oxidative stress tolerance of cells (16, 35, 36). Amino acids 4 to 191 containing the leucine zipper domain were sufficient for Rbg1 binding,

suggesting that this domain may be involved in the interaction with Rbg1 (Fig. 3).

In contrast to Yap1, the biological role of the other identified Rbg1 interaction proteins is unclear. Four identical *YGR250c* clones encoding the majority of the protein (aa 114 to 767) were isolated. Interaction between Rbg1 and Ygr250c was also reported in a genomewide two-hybrid study (26). Ygr250c is a cytoplasmic protein (23) that possesses three RNA-binding motifs. The function of Ygr250c is unknown and deletion of *YGR250c* resulted in no detectable phenotype (50; data not shown).

We obtained 14 overlapping fragments of Tma46 (Fig. 3), a protein of unknown function that was previously reported to be in a complex with Rbg1 (10, 33). The smallest Rbg1-interaction domain of Tma46 consisted of 42 aa (i.e., aa 254 to 296), suggesting that this portion contains the Rbg1 binding site. Tma46 has a putative zinc finger (aa 88 to 113) and has been identified as a protein associated with the nuclear pore complex (49, 64). Tma46 is also reported to associate with polysomes (10).

Thirteen identical *YDR152w* clones (aa 167 to 265) were isolated as encoding Rbg1 interacting partners (Fig. 3), suggesting that Rbg1 interacts with the C terminus of Ydr152w. Ydr152w was previously identified as an interacting partner of both yeast DRG proteins, Rbg1 and Rbg2, in genomewide two-hybrid and protein-protein interaction studies (25, 33, 64). It was demonstrated that *YDR152w* has a synthetic interaction with ribosomal genes and, therefore, *YDR152w* was renamed *GIR2* for “genetically interacts with ribosomal genes” (Krogan, unpublished). Although the function of Gir2 is unknown, the N terminus (aa 5 to 156) shares sequence similarity to the N-terminal GI domain of Gcn2 (34), a polyribosome-associated protein (47). Interestingly, native Gir2 seems to be highly unstructured, perhaps only adopting a folded conformation in the presence of binding partners (1, 2).

**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

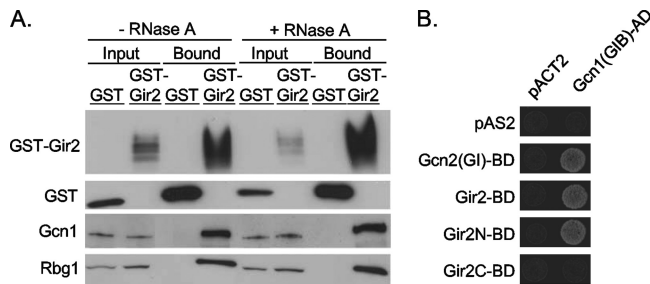


FIG. 4. Association of Gir2 with Gcn1 and Rbg1. (A) Copurification of Rbg1 and Gcn1 with GST-Gir2. GST and GST-Gir2 were affinity-purified with glutathione-Sepharose beads from yeast strains (H1511) expressing plasmid-borne GST alone or GST-Gir2 from a galactose-inducible promoter (pES128-9-1 and pJM3958, respectively). Aliquots of cellular extract, treated with or without RNase A, loaded onto the beads (input) or from the affinity-purified (bound) fractions, were analyzed by immunoblotting with antibodies to GST, Rbg1, or Gcn1, as indicated. (B) Cells expressing the GAL4 activation domain alone (pACT2) or fused to Gcn1(GIB)-AD [pACT2-GCN1(GIB)] and either the GAL4-binding domain alone (pAS2) or fused to Gcn2(GI), Gir2, Gir2N, or Gir2C [pAS-GCN2(GI), pAS-GIR2, pAS-GIR2N, and pAS-GIR2C, respectively] were analyzed for expression of *HIS3* reporter gene by growth on SD-His-Trp-Leu plus 3-AT.

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 Gcn2 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 biosyn-

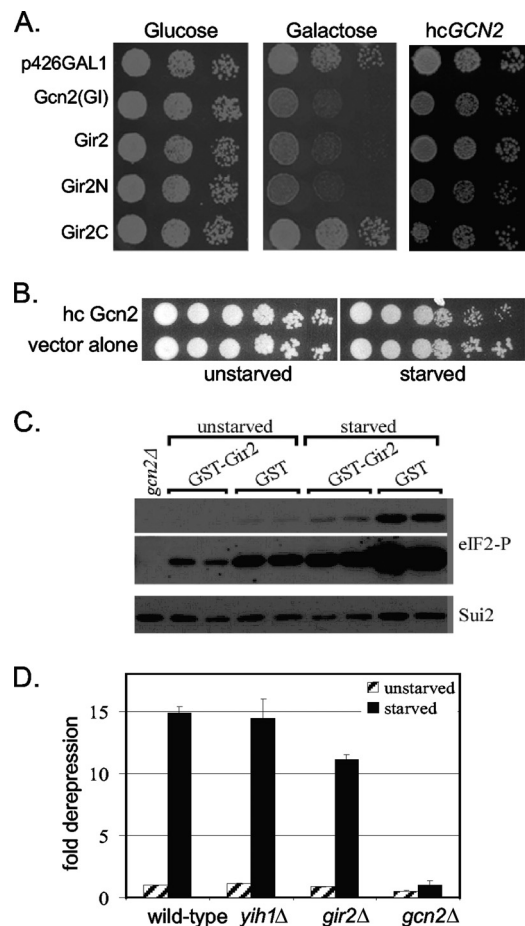


FIG. 5. Gir2 expression and amino acid control. (A) Overexpression of Gir2 leads to growth inhibition on 3-AT. Tenfold serial dilutions of cells harboring vector alone (p426-GAL1) or expressing *GCN2*(GI), *GIR2*, *GIR2N*, or *GIR2C* under the GAL1 promoter were spotted on plates containing either glucose or galactose, as indicated, in the presence of 10 mM 3-AT and incubated at 30°C. Similar strains that also express Gcn2 from a high-copy plasmid (hcGCN2; pAH15) were grown in the presence of galactose and 10 mM 3-AT. (B) Overexpression of hcGCN2 slightly perturbs the growth of starved cells. Tenfold serial dilutions of wild-type cells harboring hcGCN2 or vector alone in the absence (unstarved) or presence (starved) of 10 mM 3-AT. (C) Yeast strains (H1511) expressing plasmid-borne GST alone or GST-Gir2 and the isogenic *gcn2Δ* strain H2557 were grown to exponential phase and harvested, followed by immunoblot analysis with antibodies to eIF-2 $\alpha$  and phosphorylated eIF-2 $\alpha$ . Two different exposure times for phosphorylated eIF-2 $\alpha$  are shown (two panels linked together by a gray bar on the right-hand side, with the upper panel showing short exposure and the lower panel showing long exposure). (D) Expression of *GCN4-lacZ*. Expression of *GCN4* was determined in the strains indicated using a *lacZ*-based reported construct (p180), and the fold derepression was determined as the ratio of the wild-type values under nonstarvation conditions. For each strain, at least two independent transformants were assayed, in duplicate (*gcn2Δ* strain) or at least in triplicate, and the average values as well as the standard error values are shown in the graph.

thetic enzyme encoded by *HIS3*. 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 damp-

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-2 $\alpha$ , even under amino acid-replete conditions. To test our prediction, we examined the phosphorylation state of eIF-2 $\alpha$  in strains overexpressing GST-Gir2. As expected, the levels of eIF-2 $\alpha$  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 *gir2* $\Delta$  mutant had an effect on the output of a Gcn2-regulated gene, *GCN4*. Isogenic wild-type, *yih1* $\Delta$ , *gir2* $\Delta$ , and *gcn2* $\Delta$  strains were transformed with a plasmid (p180) harboring a *GCN4-lacZ* reporter construct in which the translation of  $\beta$ -galactosidase is under the control of the *GCN4* 5' untranslated region containing four upstream open reading frames, and  $\beta$ -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 dele-

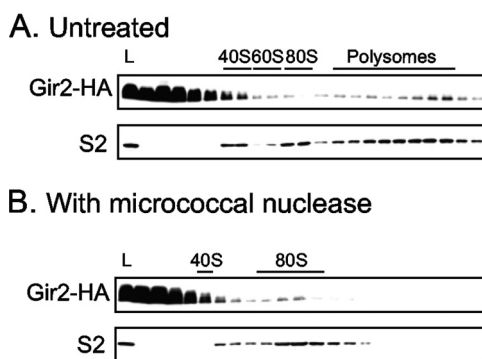


FIG. 6. Gir2 partially cofractionates with translating ribosomes. Extracts from cells expressing Gir2-HA from the chromosome without (A) or with (B) the addition of micrococcal nuclease (5 U/ $\mu$ l) prior to fractionation on sucrose gradients. Immunoblots of indicated fractions reveal the positions of Gir2-HA and ribosomal protein S2. The positions of the 40S, 60S, and 80S monosomes and the polysomes are labeled. L, 1/100 extract loaded on gradient.

tion of *YIH1* did not lead to increased eIF-2 $\alpha$  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 polysomes 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

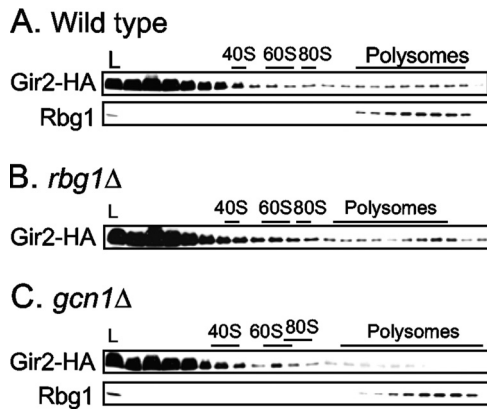


FIG. 7. Gir2 association with polysomes is partially dependent on Gcn1. Cell extracts from a wild-type strain (A), an *rbg1*Δ strain (B), or a *gcn1*Δ strain (C) were resolved by velocity sedimentation in sucrose gradients, and the positions of Gir2-HA and Rbg1 were detected by immunoblot analysis, as indicated. The positions of the 40S, 60S, and 80S monosomes and the polysomes are indicated. L, 1/100 extract loaded on gradient.

sucrose density gradients does not significantly differ from that seen in wild-type extracts (Fig. 7B compared to Fig. 7A), suggesting that Gir2-3HA associates with the polysomes independently of Rbg1. In the absence of Gcn1, however, there was a dramatic decrease in the amount of Gir2-3HA associated with polyribosomes (Fig. 7C). Furthermore, the polyribosome association 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 members of the Obg/CgtA family (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

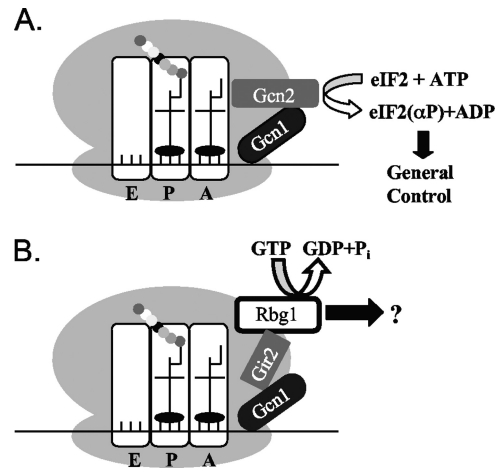


FIG. 8. Modeling the interaction between translating ribosomes and Gcn2, Gcn1, Rbg1, and Gir2. (A) Gcn1 interacts with Gcn2 on the ribosome for detecting uncharged tRNAs under amino acid starvation. This leads to the stimulation of the Gcn2 kinase domain, subsequent phosphorylation of its substrate eIF-2 $\alpha$ , and finally to the activation of the general amino acid control (see references 42 and 53). (B) On the ribosome, Gir2 binds simultaneously Rbg1 via its C-terminal domain, and Gcn1 binds via its N-terminal domain. In a manner analogous to the general amino 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.

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



acid starvation) is analogous to the Gcn1/Gcn2 response in *S. cerevisiae* (42, 53). RelA, a polysome-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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