The general amino acid control (GAAC) enables yeast cells to overcome amino acid deprivation by activation of the α subunit of translation initiation factor 2 (eIF2α) kinase GCN2 and consequent induction of GCN4, a transcriptional activator of amino acid biosynthetic genes. Binding of GCN2 to GCN1 is required for stimulation of GCN2 kinase activity by uncharged tRNA in starved cells. Here we show that YIH1, when overexpressed, dampens the GAAC response (Gcn− phenotype) by suppressing eIF2α phosphorylation by GCN2. The overexpressed YIH1 binds GCN1 and reduces GCN1-GCN2 complex formation, and, consistent with this, the Gcn− phenotype produced by YIH1 overexpression is suppressed by GCN2 overexpression. YIH1 interacts with the same GCN1 fragment that binds GCN2, and this YIH1-GCN1 interaction requires Arg-2259 in GCN1 in vitro and in full-length GCN1 in vivo, as found for GCN2-GCN1 interaction. However, deletion of YIH1 does not increase eIF2α phosphorylation or derepress the GAAC, suggesting that YIH1 at native levels is not a general inhibitor of GCN2 activity. We discovered that YIH1 normally resides in a complex with monomeric actin, rather than GCN1, and that a genetic reduction in actin levels decreases the GAAC response. This Gcn− phenotype was partially suppressed by deletion of YIH1, consistent with YIH1-mediated inhibition of GCN2 in actin-deficient cells. We suggest that YIH1 resides in a YIH1-actin complex and may be released for inhibition of GCN2 and stimulation of protein synthesis under specialized conditions or in a restricted cellular compartment in which YIH1 is displaced from monomeric actin.

Phosphorylation of the α subunit of translation initiation factor 2 (eIF2α) is a key regulatory mechanism for down-regulating protein synthesis in response to starvation or stress in eukaryotic cells. Four different eIF2α kinases have been identified in mammalian cells that are activated by different stimuli: HRI by hemin deprivation (1), PKR by double-stranded RNA (2), PERK or PKR by unfolded proteins in the endoplasmic reticulum (3), and GCN2 by amino acid starvation (4, 5). eIF2α is necessary for delivery of charged initiator methionyl-tRNA (Met-tRNA^Met) to the 40 S ribosomal subunits in an eIF2-GTP-Met-tRNA^Met ternary complex and is released from the initiation complex in the inactive GDP-bound form. Phosphorylation of eIF2α at serine 51 by eIF2α kinases converts eIF2α from a substrate to an inhibitor of its guanine nucleotide exchange factor, eIF2B. Since only eIF2-GTP can bind Met-tRNA^Met, the inhibition of eIF2B evoked by eIF2α phosphorylation leads to a decrease in ternary complex formation and a general reduction in protein synthesis (4).

GCN2 is the sole eIF2α kinase in the yeast *Saccharomyces cerevisiae*, where it was first identified as being required for growth under amino acid starvation conditions. Phosphorylation of eIF2α by GCN2 induces the translation of *GCN4* mRNA, encoding a transcriptional activator of amino acid biosynthetic enzymes, while dampening the translation of most other messages. Four short open reading frames in the *GCN4* mRNA leader underlie a specialized reinitiation mechanism that allows efficient translation of this mRNA when the ternary complex level drops (4). The increased expression of *GCN4* and its amino acid biosynthetic target genes in starved cells is known as general amino acid control (GAAC) (6).

GCN2 is present as a latent eIF2α kinase in the yeast. The uncharged tRNA binds to a regulatory domain in GCN2 that resembles histidyl-tRNA synthetase, and this interaction is believed to induce a conformational change that overcomes an intrinsic defect in the adjacent kinase domain (7–10). The products of GCN1 and GCN20 are also necessary for the activation of GCN2 by uncharged tRNA in starved cells (11, 12). GCN1 and GCN20 form a protein complex (12) that binds to the N-terminal domain (NTD) of GCN2, which is highly conserved among all GCN2 orthologs. The GCN2 NTD is required for kinase function in vivo, and its overexpression impairs complex formation between native GCN1 and GCN2 and produces a dominant Gcn− (general control noninducible) phenotype (13, 14). A C-terminal
Overexpressed Actin-binding Protein YIH1 Inhibits GCN2

TABLE I
Strains used in this study

| Strain          | Genotype                                                                 | Source                        |
|-----------------|--------------------------------------------------------------------------|-------------------------------|
| Genetic background: H1511 | MATα ura3-52 trp1-63 leu2-3,112 GAL2+                             | Ref. 43                        |
| H1511           | MATα ura3-52 trp1-63 leu2-3,112 GAL2+ gen1D                             | C. R. Vazquez de Aldana and A. G. Hinnebusch |
| Genetic background: H1402 | MATα inol ura3-52 leu2-3,112 (HIS4::lacZ ura3-52)          | Ref. 44                        |
| H1402           | As H1402 and GCN2::E601K-E1591K                                         | Ref. 33                        |
| Genetic background: BY4741 | MATα his3Δ1 leu2Δ0 met15Δ1 ura3Δ0                                   | Research Genetics              |
| BY4741          | As BY4741 and yih1Δ::Kan<sup>+</sup>                                  | Research Genetics              |
| 5780            | As BY4741 and gen1Δ::Kan<sup>+</sup>                                  | Research Genetics              |
| 4562            | As BY4741 and gen1Δ::Kan<sup>+</sup>                                  | Research Genetics              |
| 5688            | As BY4741 and gen20Δ::Kan<sup>+</sup>                                  | Research Genetics              |
| 249             | As BY4741 and gen42Δ::Kan<sup>+</sup>                                  | This study                     |
| MSY-WT2         | MATα leu2Δ0 met15Δ1 ura3Δ0                                            | This study                     |
| ESY-11b         | As MSY-WT2 and encoding FLAG tag 5' of YIH1                           | This study                     |
| MSY-Y2          | As MSY-WT2 and yih1Δ::Kan<sup>+</sup>                                  | This study                     |
| MSY-1–1         | As MSY-WT2 and gen1Δ::Kan<sup>+</sup>                                  | This study                     |
| EMSY8053-3-1    | As MSY-WT2 and gen20Δ::HisG                                            | This study                     |
| MSY-20-1        | As MSY-WT2 and gen20Δ::Kan<sup>+</sup>                                  | This study                     |
| MSY-4           | As MSY-WT2 and gen4Δ::Kan<sup>+</sup>                                  | This study                     |
| Genetic background: BY4743 | MATα/MATα his3αΔ1 his3αΔ1 leu2Δ0/0 ura3Δ0/0 ura3Δ0 met15Δ1/15Δ1 lys2Δ0/LYS2 | Research Genetics              |
| BY4743          | As BY4743 and yih1Δ::HisG/1Δ::loxP                                    | This study                     |
| ESY12A-a        | As BY4743 and yih1Δ::hisG/1Δ::loxP                                    | This study                     |
| ESYE6-b         | As BY4743 and yih1Δ::hisG/1Δ::loxP                                    | This study                     |
| 33642           | As BY4743 and gen2Δ::Kan<sup>+</sup>gen20Δ::Kan<sup>+</sup>             | Research Genetics              |
| 27075           | As BY4743 and act1Δ::Kan<sup>+</sup>/ACT1                               | Research Genetics              |

MATERIALS AND METHODS

Strains and Plasmids—Yeast strains and plasmids used in this study are summarized in Tables I and II, respectively. Details of their construction are as follows. To generate HIS3<sup>+</sup> versions of strains BY4741, 5780, 4562, 5688, and 249, the wild type HIS3 gene was excised from plasmid pRS415 (21) as an Eco47III/NsiI fragment and used to transform each strain to His<sup>+</sup>. The conversion of his3α to HIS3 was verified by PCR amplification of genomic DNA (22) using primer his3a, annealing to sequences within the Eco47III/NsiI fragment (5'GAG AAA GTA GAA GAT CTC TCT TGC G-3'), and primer his3b, annealing to sequences near the HIS3 gene outside of this fragment (5'G418 CCG GCC GGC TCG TCT AGA ATG ACA C-3').

For construction of yeast strain ESY-11b, the yih1Δ::Kan<sup>+</sup> strain MSY-Y2 was transformed with SpeI-digested pES197-1, and the resulting transformants were grown on 5-fluoroorotic acid medium to select for evolution of the plasmid. A Ura<sup>-</sup> G418-sensitive transformant was isolated, and the replacement of yih1Δ::Kan<sup>+</sup> with FLAG-YIH1 was verified by PCR amplification of genomic DNA using the appropriate primers.

A strain MSY-WT2 with EcoRI- and XbaI-digested plasmid pHQ1093, containing the gen2Δ::hisG/URA3::hisG disruption cassette. Expression of the URA3 marker was selected by growth on 5-fluoroorotic acid medium, and deletion of GCN2 was verified by complementation tests with plasmid-borne GCN2.
For deletion of both YHI1 alleles in wild-type BY4743 and the ACT1/act11 heterozygote 27075, XbaI- and Sail-digested pES222-202-2 was introduced into these strains. Transformants in which one YHI1 allele was replaced by the hisG::URA3::hisG cassette were transformed with the box:LEU2::loxP construct harboring YHI1 flanking sequences, generated by PCR according to Gueldener et al. (23) using primers ES400-34B and ES400-35B containing 81/80 nucleotides upstream/downstream of YHI1 and template pUG73. The LEU2 marker was evicted via introduction of the Cre expression plasmid pSH65 (23), and the URA3 marker was evicted by selection on 5-fluoroorotic acid medium. Successful replacement of the YHI1 alleles by the respective selective markers in the resulting yeast strains was verified by PCR analysis using primers that flank the YHI1 open reading frame.

The ACT1 gene and its promoter were amplified from yeast strain BY4741 using primers designed to contain EcoRI sites that anneal to sequences upstream (Act1-UP, 5′-CAC AGG ATC TAA CAT AAT ACA CAC GCC AGA AC-3′) and downstream (Act1-DOWN, 5′-GAG AGG AAT TAA CAT GAT ACA CGG TCC AAC GGA GG-3′) of the ACT1 coding region. The PCR product was digested with EcoRI and cloned into the EcoRI site of YEp24 (24) to create plasmid pMJS1. For construction of plasmids expressing the YHI1 gene from a heterologous promoter, the YHI1 open reading frame was PCR-amplified from yeast strain BY4741 using primer ES400-4 (5′-GCC GCG TCG ACT ACC TAG GCC GCG TCG TGG CTT TTA GGC ACC TTG TGC AGT CAT ATA CGC TG-3′) and downstream primer ES400-5 (TC CAT TGA CCT TTT CCT TCC TC), containing at bases 4–9 the unique BglII site in YHI1 and sense primer ES200-1 (GCG GCG TCG ACT ACC TAG GCC GCG TCG TGG CTT TTA GGC ACC TTG TGC AGT CAT ATA CGC TG), containing at bases 1–17 from immediately upstream of YHI1, at bases 18–20 the start codon, and at bases 21–38 the six first codons of the FLAG tag. Two separate PCR products were performed, one amplifying the sequence extending from the upstream cloning site NotI to the YHI1 ATG using primers ES400-1 and ES400-5 and pES185-1 as template and the second reaction amplifying sequences extending from the YHI1 ATG to the BiGII site in the YHI1 gene with primers ES400-6 and ES400-8, using pES191-H1 as template. The two PCR products were fused together by performing a third PCR with primers ES400-1 and ES400-4 and the products of the first two PCR reactions as template. The resulting PCR fragment was digested with NotI and BiGII and used to replace the NotI-BiGII fragment in pES185-1, resulting in pES186-4. The Sall-SacI fragment of pES186-4, containing the FLAG-YHI1 gene including surrounding sequences, was introduced into YEp24 digested with the same enzymes (24), resulting in pES197-1.

For construction of pES200-2, the Mlu-Sall fragment of p1832 was used to replace the Mlu-Sall fragment excised from pES174-3 (15). The YHI1::hisG::URA3::hisG deletion cassette was constructed as follows. A 100-bp fragment immediately upstream of YHI1 was PCR-amplified from yeast strain BY4741 using primers ES400-2 and ES400-23. Bases 6–11 of ES400-22 introduce a XbaI site, and bases 12–32 correspond to bases 83–103 upstream of YHI1 (CCG GCT GTA TAC TGA TTG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site in YHI1 (TCA TAA TAT GAT CTT GTG TGG TGC AGT CAT ACA GGC TGG). Bases 15–36 anneal to the 22 bases immediately upstream of YHI1 (TCA TAA TAT GAT CTT GTG TGG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site, and bases 15–36 anneal to the 22 bases immediately upstream of YHI1 (TCA TAA TAT GAT CTT GTG TGG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site, and bases 15–36 anneal to the 22 bases immediately downstream of YHI1, and bases 1–22 are complementary to bases 1–22 of primer ES400-23 (AAA GCT CAA GAT CTC ATT ATG ATT ATG ATG TCA AGC ACC). Bases 6–11 of primer ES200-2 introduce a Sall site, and bases 12–32 anneal to bases 110–131 downstream of YHI1 (GCC GCG TCG ACT ACC TAG AAC ATT ATG ATG TTC TTA TTA GGC ACC TGG). By a subsequent PCR using both PCR-amplified fragments just described as templates and primers ES400-22 and ES400-25, the 100-bp sequence upstream and 130-bp sequence downstream of YHI1 were fused together and separated by only a BiGII site. The fusion product was digested with XbaI and Sail and introduced into the similarly digested plasmid pBluescript (Stratagene), yielding pES221-6. The hisG::URA3::hisG cassette was excised from

---

**Table II**

| Plasmid | Gene* | Selectable marker | Vector | Source |
|---------|-------|------------------|--------|--------|
| pAH15   | GCN2  | LEU2, 2 µ         | YEp13  | Ref. 45 |
| pG12    | GCN2::M788V | URA3, CEN6/ARSH4 | pRS316 | This study |
| p1832   | GCN1-myc  | LEU2, CEN6/ARSH4 | pRS315 | This study |
| pES196-6-4 | FLAC::YHI1 | URA3, CEN6/ARSH4 | pRS316 | This study |
| pES200-2-2 | GenR::T225B::myc  | URA3, CEN6/ARSH4 | pRS315 | This study |
| pES222-202-2 | YHI1::hisG::URA3::hisG cassette | URA3 | pBluescript | This study |
| pES197-1 | ACT1  | URA3, ARS1/CEN4 | YEp2444 | This study |
| pMJS1   |       |                  |        |        |

* Numbers in parentheses indicate amino acids encoded by the respective gene.

**Bacterial gene fusions**

| pES171-H1    | His6-gcn2(1–598) | Kan         | pET-28a | Ref. 15 |
| pES189-D1A  | His6-gcn1(1–592) | Kan         | pET-28a | This study |
| pES123-B1   | GST-gcn1(1–2052–2428) | Amp         | pGEX-6p-3 | Ref. 15 |
| pES164-2A   | GST-gcn1(1–2051–2428–R2259A | Amp         | pGEX-6p-2 | Ref. 15 |

**Yeast gene fusions, under GALI-CYC1 promoter (all AmpR)**

| pES187-B1 | GST::YHI1 | URA3, leu2a, 2 µ | pES128-9-1 | This study |
| pES191-H1 | FLAG::YHI1 | URA3, leu2a, 2 µ | pES128-9-1 | This study |

---

* A. G. Hinnebusch, unpublished observations.
* Constitutively active GCN2 allele.
* Epitope tag at the C terminus of the open reading frame.

---

**Overexpressed Actin-binding Protein YIH1 Inhibits GCN2**

G. Pavitt and A. G. Hinnebusch, unpublished observations.

---

The YHI1::hisG::URA3::hisG deletion cassette was constructed as follows. A 100-bp fragment immediately upstream of YHI1 was PCR-amplified from yeast strain BY4741 using primers ES400-2 and ES400-23. Bases 6–11 of ES400-22 introduce a XbaI site, and bases 12–32 correspond to bases 83–103 upstream of YHI1 (CCG GCT GTA TAC TGA TTG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site in YHI1 (TCA TAA TAT GAT CTT GTG TGG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site, and bases 15–36 anneal to the 22 bases immediately upstream of YHI1 (TCA TAA TAT GAT CTT GTG TGG TGC AGT CAT ACA GGC TGG). Bases 9–14 of ES400-23 introduce a BglII site, and bases 15–36 anneal to the 22 bases immediately downstream of YHI1, and bases 1–22 are complementary to bases 1–22 of primer ES400-23 (AAA GCT CAA GAT CTC ATT ATG ATG TCA ATG TCA AGC ACC). Bases 6–11 of primer ES200-2 introduce a Sall site, and bases 12–32 anneal to bases 110–131 downstream of YHI1 (GCC GCG TCG ACT ACC TAG AAC ATT ATG ATG TTC TTA TTA GGC ACC TGG). By a subsequent PCR using both PCR-amplified fragments just described as templates and primers ES400-22 and ES400-25, the 100-bp sequence upstream and 130-bp sequence downstream of YHI1 were fused together and separated by only a BiGII site. The fusion product was digested with XbaI and Sail and introduced into the similarly digested plasmid pBluescript (Stratagene), yielding pES221-6. The hisG::URA3::hisG cassette was excised from
plasmid pHQ221 with BamHI and inserted into BglII-digested pES221-6, resulting in pES222-209.

Quick Preparation of Genomic DNA from Yeast Cells for PCRs—Cell pellets from 5 ml of yeast overnight cultures were resuspended in 200 μl of TE buffer. 100 μl of glass beads (0.5-mm diameter) and 200 μl of phenol were added, and the samples were vortexed for 2 min. The supernatant was extracted once with 200 μl of chloroform and then used directly as a template in PCRs.

Protein Techniques—In vitro and in vivo binding assays using GST and His fusion proteins and co-immunoprecipitation and GST pull-down assays were performed as described previously (15). Proteins were separated by SDS-PAGE using gradient gels (4–12 or 4–20%; Invitrogen). Proteins were visualized in gels by staining with Coomassie R250 (0.1% w/v) in 40% ethanol and 10% acetic acid and subsequent treatment with destain solution I (20% ethanol, 7% acetic acid) and destain solution II (10% ethanol, 5% acetic acid). For Western blot analysis, proteins were transferred to nitrocellulose membranes (Invitrogen) according to the manufacturer’s protocol, and proteins on the membranes were stained with Ponceau S (0.5% w/v, in 1% acetic acid) according to standard procedures (26). Proteins were detected by the enhanced chemiluminescence detection system (Amersham Biosciences) using antibodies against GCN1 (HL1405, dilution 1:1000 (12)), GCN2 (HL2523, 1:1000 (27)), eIF2α (1:2000 (28)), eIF2β phosphorylated on Ser-51 (1:5000; BioSource International, Inc.), actin (1:5000 (29)), His6 (1.500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and FLAG (1:500; Sigma). Immune complexes were visualized using horseradish peroxidase conjugated to donkey anti-rabbit antibodies, to sheep anti-mouse antibodies (for detection of flag antibodies), or to protein A (for detection of actin antibodies) (Amersham Biosciences).

Purification of the YIH1-containing Complex—Yeast strains were grown in YPD medium to an exponential phase (A600 = 10) in a 10-liter fermenter and harvested via continuous flow centrifugation, added in buffer A (1× phosphate-buffered saline, 10% glycerol, 1× phenylmethylsulfonyl fluoride, 0.5× mercuric thiocarboxylate, 1× complete protease inhibitor tablets (Roche Applied Science) to about 1 g of cells/ml, and passed twice through a French pressure cell. Cell debris was removed (10,500 rpm, 15 min), and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h). Incubation of the extract with 500 μl of cells/ml, and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h). Incubation of the extract with 500 μl of cells/ml, and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h). Incubation of the extract with 500 μl of cells/ml, and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h). Incubation of the extract with 500 μl of cells/ml, and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h). Incubation of the extract with 500 μl of cells/ml, and the extract was cleared by centrifugation (Beckman, 100,000 g, 4 h).

RESULTS

Overexpressed YIH1 Competes with GCN2 for GCN1 Binding in Vivo—Binding of the GCN2 NTD to amino acids 2052–2427 (region D) of GCN1 is essential for activation of GCN2 under amino acid starvation conditions (15). YIH1 can bind to the same region of GCN1 in the yeast two-hybrid assay, and overexpression of YIH1 produced a dominant Gcn7 phenotype, manifested by sensitivity to 3AT (14). These findings suggested that YIH1, if overexpressed, can compete with GCN2 for GCN1 binding and prevent activation of GCN2 in cells starved for histidine with 3AT. If so, then overexpression of GCN2 should overcome this competition and suppress the Gcn7 phenotype of overexpressing YIH1. To test this prediction, we added a GST or FLAG tag at the N terminus of the YIH1 open reading frame and placed it under a galactose-inducible promoter. As reported, overexpression of the tagged YIH1 alleles conferred dominant 3AT sensitivity (3AT+) in an otherwise wild type strain (Fig. 1, A and B). Importantly, overexpression of GCN2 from a high copy plasmid (hcGCN2) suppressed the Gcn7 phenotype associated with GST-YIH1 overexpression (Fig. 1B), consistent with the hypothesis that YIH1 impairs GAAC by competing with GCN2 for the same binding site on GCN1.

To provide additional proof for this conclusion, we took advantage of the fact that a constitutively active GCN2 protein encoded by GCN2*/E601K-E1591K (33) causes a slow growth (Slg−) phenotype resulting from hyperphosphorylation of eIF2α and the consequent general inhibition of protein synthesis. The Sgr− phenotype of GCN2*/E601K-E1591K is suppressed by deletion of GCN1 (33), suggesting that GCN2*/E601K-E1591K requires GCN1 binding for its activation. Therefore, if overexpressed YIH1 disrupts GCN1-GCN2 interaction, it should reverse the Sgr− phenotype associated with the GCN2* allele. In accordance with this prediction, overexpression of GST-YIH1 suppressed the Sgr− phenotype of GCN2*/E601K-E1591K (Fig. 1C). Taken together, these data provide strong genetic evidence that overexpressed YIH1 impairs activation of GCN2 and blocks the GAAC response by competing with GCN2 for binding to GCN1.

Thus far, YIH1-GCN1 interaction has been demonstrated by the yeast two-hybrid assay and with an in vitro binding assay using recombinant GCN1 fragments encompassing the GCN2- YIH1 binding site (16). To provide physical evidence that overexpressed YIH1 sequesters native GCN1 in vivo, we asked whether endogenous GCN1 can be co-precipitated with GST-YIH1 on glutathione-agarose beads from extracts of yeast cells overexpressing GST-YIH1. We found that GCN1, but not GCN2, coprecipitated specifically with GST-YIH1 (Fig 2A), providing evidence that overexpressed GST-YIH1 binds to native GCN1 but not to the GCN1-GCN2 complex in vivo. This conclusion is consistent with the idea that YIH1 competes with GCN2 for GCN1 binding.

Next, we sought physical evidence that overexpressing YIH1 reduces GCN1-GCN2 complex formation in vivo. Cell extracts of strains overexpressing GST-tagged YIH1 or GST alone were immunoprecipitated with GCN1 antibodies, and the immune complexes were probed with GCN1 and GCN2 antibodies. The results in Fig. 2B indicated that about 53% of cellular GCN2 was complexed with GCN1 in the strain expressing GST alone, in agreement with our previous results (15). When GST-YIH1 was overexpressed in the cell, the amount of GCN2 associated with GCN1 was reduced by almost one-half (28% of cellular GCN2 complexed with GCN1), demonstrating that overexpressing YIH1 reduces GCN1-GCN2 complex formation in vivo.

It was important to establish that the Gcn7 phenotype of high copy YIH1 results from decreased phosphorylation of eIF2α on Ser-51, the site of phosphorylation by GCN2. In accordance with this expectation, we found that YIH1 overexpression reduced the levels of eIF2α phosphorylated on Ser-51 (eIF2α-P) by ~40% under starvation conditions and by about 90% in nonstarved cells (Fig. 2C). Together, the results in Figs. 1 and 2 show that overexpressed YIH1 inhibits GCN2 kinase activity by disrupting GCN1-GCN2 interaction.

Arg-2259 in GCN1 Is Essential for YIH1 Binding in Vitro and in Vivo—We showed previously that substitution of Arg-2259 in GCN1 abolished association of native GCN1 and GCN2 in vivo and impaired interaction of the recombinant N terminus of GCN2 with region D of GCN1 in vitro (15). The fact that...
the binding sites for YIH1 and GCN2 mapped to almost the same region in GCN1 (14, 15) suggests that GCN2 and YIH1 might interact with the same residues in GCN1. To test whether YIH1 also requires Arg-2259 for GCN1 interaction, we tested a recombinant His<sub>6</sub>-YIH1 fusion protein expressed in E. coli for interaction with bacterially expressed GST-GCN1-2428, which contains the minimal GCN1 fragment sufficient for GCN2 binding (15). We found that GST-GCN1-2428 is sufficient for binding to His<sub>6</sub>-YIH1 in vitro and that this interaction was abolished by the R2259A mutation in the GCN1 fragment (Fig. 3A). In order to show that Arg-2259 in native GCN1 is essential for YIH1 binding in vivo, we performed GST pull-down assays on extracts of gen<sup>1</sup>Δ strains overexpressing GST-YIH1 and containing either GCN1 or gen<sup>1</sup>-R2259A on a low copy plasmid. As shown in Fig. 3B, GCN1, but not genl-R2259A, coprecipitated with GST-YIH1 from cell extracts. These findings suggest that YIH1 and GCN2 share a key binding determinant in GCN1 region D, supporting the idea that they compete directly for complex formation with YIH1.

Is YIH1 a Negative Regulator of the GAAC When Expressed at Native Levels?—The results presented above suggest that YIH1 is a negative regulator of GCN2 that, presumably, must be disabled to permit a strong GAAC response in starved cells. If so, then deletion of YIH1 should lead to constitutive activation of GCN2. To test this possibility, we asked whether deletion of YIH1 would increase the level of eIF2α phosphorylation in nonstarvation conditions or decrease the time required for eIF2α-P to return to the basal level following the addition of histidine to 3AT-starved cells. We saw no increase in the level of eIF2α-P in yih1Δ versus wild-type cells under nonstarvation conditions (Fig. 5A, lanes 1–4 versus lanes 5 and 6) or in cells starved for histidine or glutamine by the addition of the antimetabolites 3AT or l-methionine-S-sulfoximine (MSX), respectively (Fig. 5B). In addition, we found that eIF2α-P levels declined at indistinguishable rates in wild-type and yih1Δ cells, returning to the basal level within 6 min in both strains (Fig. 5C). The experiments described above were carried out using synthetic medium supplemented with all 20 amino acids as the nonstarvation condition (data not shown). Thus, there is no evidence that YIH1 is required to prevent GCN2 activation in nonstarvation conditions, to limit the extent of GCN2 activation in starved cells, or to increase the rate at
Fig. 2. Overexpressed YIH1 binds to GCN1 and actin, reduces GCN1-GCN2 association, and lowers eIF2α phosphorylation in vivo. A, overexpressed YIH1 binds to GCN1 and actin in vivo, but not to GCN2. Transformants of wild-type strain H1511 containing the galactose-inducible genes GST-YIH1 (on pES187-B1) or GST alone (on pES128-9-1), respectively, were grown in minimal medium containing galactose as carbon source. WCEs were prepared, and aliquots with equivalent amounts of protein were subjected to GST pull-down assays using glutathione-Sepharose beads. The precipitated complexes were probed with antibodies against GCN1, GCN2, and actin in Western blots (upper three panels). The bottom panel shows Ponceau S staining of the membrane, revealing the amounts of GST-YIH1 and GST that were precipitated. The input lanes contained 10% of the WCEs used in the assays; the pellet lanes contained 100% of the precipitated proteins. B, overexpressed YIH1 reduces GCN1-GCN2 complex formation in vivo. WCEs from gcn1Δ strain H2556, harboring the galactose-inducible gene GST-YIH1, and GCN1 strain H1511, containing GST-YIH1 or GST alone, respectively, were prepared as outlined in A and immunoprecipitated with GCN1 antibodies. The immune complexes were probed for GCN2 and GCN1 in Western blots (upper two panels). 10% of input (I) and supernatant (S) and 100% of precipitated protein (P) were loaded on the gel. The histogram shows the average percentage of GCN2 immunoprecipitated after normalizing for the amount of GCN1 that immunoprecipitated from the same samples (set to 100%). The mean values and S.E. values (shown by error bars) were calculated from at least eight independent assays. C, overexpressed YIH1 reduces eIF2α-P levels under starvation and nonstarvation conditions. Yeast strains described in A were grown to exponential phase in medium containing galactose and then starved for histidine by the addition of 3AT (20 mM) for 4 h. WCEs of these cultures were subjected to Western blot analysis, using antibodies against eIF2α (eIF2α-P) and antibodies against actin, respectively. Two different exposures are shown for the eIF2α-P detection. The Western signals from two independent strains and two independent blot samples were quantified using NIH Image software, and the results and S.E. values are presented at the bottom as the mean eIF2α-P to total eIF2α ratios relative to the corresponding mean ratio measured for the unstarved GST control (lanes 3 and 4).

which eIF2α phosphorylation declines to basal levels when starved cells are replenished with the limiting amino acid.

The yeast genome encodes two other proteins containing domains related to the GCN1-binding domains in GCN2 and YIH1, known as YDR152W and YLR419W. We considered the possibility that YIH1 is functionally redundant with one of these predicted proteins for down-regulation of GCN2. To test this idea, we examined strains deleted for these genes singly or in combination with yih1Δ for resistance to 5FT and TRA. The results identified unambiguously the copurifying protein as YIH1 (Fig. 6, A and B). The copurifying protein was excised from the gel and subjected to trypsin digestion, and the recovered peptides were sequenced using tandem mass spectrometry. The results identified unambiguously the copurifying protein as actin, encoded by ACT1, and we confirmed this conclusion by Western blot analysis using antibodies against actin (Fig. 6, A and B). Using GCN1 antibodies, we could not detect any GCN1 co-purifying with FLAG-YIH1, suggesting that under the growth conditions used for purification of the YIH1 complex (YPD medium, native amounts of YIH1) YIH1 does not interact with GCN1, or else the YIH1-GCN1 interaction is too weak to withstand the purification procedures. We showed above that
binding of YIH1 to GCN1 is dependent on Arg-2259 in the GCN1 C-terminal region in vitro and in vivo. A, GST-GCN1-(2052-2428) directly interacts with His6-YIH1 in vitro dependent on Arg-2259. The indicated GST-GCN1 fusions (wild-type [wt] or R2259A) or GST alone encoded by plasmids pES123-B1, pES164-2A, and pGEX-6p-1, respectively, were expressed in E. coli, immobilized on glutathione-Sepharose beads, and incubated with an E. coli extract containing His6-YIH1 encoded by plasmid pES189-D1A. GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Ponceau S staining (upper panel). GST proteins were visualized via Pounce...
null
FIG. 7. Deletion of YIH1 reduces the proportion of cells stainable with rhodamine-phalloidin but does not alter the morphology of the actin cytoskeleton. A, cells were grown in YPD to exponential phase to an A600 less than 0.5 absorbance units, subjected to rhodamine-phalloidin staining (42), and visualized in a Nikon E1000 light microscope using a Sensicam QE digital CCD camera (Cooke Corp.) and a customized filter set (excitation 540/25 nm; emission 605/55 nm, beam splitter 565 nm, Chroma Technology) controlled by IP Labs software (Scanalytics Inc.). B, cells in A stained weakly and strongly by rhodamine-phalloidin were counted, and the averages and S.E. values are indicated as percentage of the total number of cells. In total, 389 wild-type cells and 758 yih1Δ cells were analyzed.

FIG. 8. Haplo-insufficiency of cellular actin confers a Gcn− phenotype that is partially dependent on YIH1. A, the act1Δ/ACT1 diploid strain and other relevant isogenic diploid strains indicated (from top to bottom: 33642, BY4743, 27075, and 35780) were tested for sensitivity to SM as described in the legend to Fig. 4B, using undiluted culture and consecutive 10-fold dilutions at 30 and 37 °C, as indicated. B, overexpression of GST-YIH1 exacerbates the SM− phenotype associated with insufficient actin levels. The act1Δ/ACT1 strain 27075 and isogenic wild-type strain BY4743, both harboring plasmid-borne galactose-inducible gene GST-YIH1 or GST alone on plasmids pES187-B1 and pES128-9-1, respectively, were analyzed for SM− phenotypes at 37 °C as in A except using medium with galactose as the sole carbon source. C, inhibition of GAAC by actin is partially mediated through YIH1. Saturation cultures were prepared from strains as indicated (from top to bottom, ESY12A-a and ESY6F-b) harboring low copy vector (pRS316), plasmid-borne low copy YIH1 (pES196–6-4), or single copy ACT1 (pMJS1). Subsequently, 5 µl of 10-fold dilutions were transferred to solid medium containing additional supplements as outlined below and incubated at 36–37 °C.
yih1Δ/yih1Δ strain compared with the ACT1/ACT1 strains shown in Fig. 8C represents a YIH1-independent component of the Gcn− defect associated with actin haplo-insufficiency.

DISCUSSION

It was shown previously that YIH1 interacts with the isolated GCN2-binding domain in GCN1 and that overexpression of YIH1 confers sensitivity to 3AT, an inhibitor of histidine biosynthesis. This finding led to the prediction that YIH1 can negatively regulate GCN2 by preventing formation of the GCN1-GCN2 complex (14). In this report, we provided several lines of evidence demonstrating that the 3AT− phenotype conferred by YIH1 overexpression results from dissociation of the GCN1-GCN2 complex and consequent impaired activation of GCN2. First, we showed that overexpression of YIH1 can suppress the growth defect conferred by a constitutively activated form of GCN2 that inhibits general translation initiation by high level phosphorylation of eIF2α (33). This result provides strong genetic evidence that YIH1 overexpression specifically inhibits GCN2 function. Biochemical evidence for this conclusion came from our finding that cells overexpressing YIH1 contain reduced levels of eIF2α-P in nonstarvation conditions and also in response to histidine starvation. Evidence that overexpressed YIH1 competes with GCN2 for GCN1 binding came from an in vivo GST pull-down experiments showing that overexpressed GST-YIH1 forms a complex with native GCN1, but not with GCN2, and from our communoprecipitation experiments showing that YIH1 overexpression reduces the level of GCN1-GCN2 complexes in vivo. We also showed that binding of YIH1 to GCN1, both in vitro and in vivo, is dependent on the same residue in GCN1 (Arg-2259) that is critical for GCN1-GCN2 interaction (15). Thus, YIH1 and GCN2 share a critical binding determinant in the C-terminal region of GCN1.

These findings point to a potential role for YIH1 as a negative regulator of GCN2 and the GAAC response. However, we were unable to demonstrate complex formation between YIH1 and GCN1 in vitro unless YIH1 was overexpressed. In addition, we did not observe any effect of deleting YIH1 on the GAAC response or on the levels of eIF2α phosphorylation under starvation or nonstarvation conditions. We considered the possibility that YIH1 could be required only to ensure a rapid dephosphorylation of eIF2α when starved cells are replenished with the limiting amino acid. However, we did not detect any difference between WT and yih1Δ cells in the rate of eIF2α dephosphorylation when 3AT-treated cells were supplied with histidine. Thus, we have no evidence that YIH1 functions as a general inhibitor of GCN2 under normal growth conditions. We also considered the possibility that the negative regulatory function of YIH1 could be carried out redundantly by one of the other predicted proteins in yeast that contain a domain related to the GCN1-binding domains in GCN2 and YIH1, namely YDR152W and YLR419W. However, deleting YDR152W and YLR419W, either together or in combination with a deletion of YIH1 had no significant effect on the GAAC response.

In an effort to uncover the function of YIH1 in vivo, we purified a FLAG-tagged YIH1 expressed from the chromosomal allele under its own promoter and found that the protein occurs in a 1:1 complex with monomeric actin containing no trace of GCN1. This finding raised the possibility that the ability of YIH1 to inhibit GCN2 might be regulated by its association with G-actin. This possibility was supported by our finding that actin haplo-insufficiency in an ACT1/act1Δ heterozygote confers sensitivity to an inhibitor of isoleucine, leucine, and valine biosynthesis (SM) and that this was exacerbated by YIH1 overexpression. This Gcn− phenotype was partially suppressed by deletion of YIH1, indicating that the defect in GAAC associated with actin insufficiency was mediated at least partly by YIH1. Thus, the reduced actin levels in the ACT1/act1Δ heterozygote may reduce the concentration of monomeric actin, leading to release of YIH1 from the YIH1-actin complex and consequent formation of the YIH1-GCN1 complex with attendant inhibition of GCN2. The residual SM− phenotype observed in the ACT1/act1Δ yih1Δ/yih1Δ strain presumably reflects a YIH1-independent reduction in the expression or activation function of GCN4 in cells with an actin insufficiency.

One difficulty with the idea that actin insufficiency leads to YIH1-mediated inhibition of GCN2 is that we have been unable to detect a reduction in eIF2α-P levels in the ACT1/act1Δ heterozygote (data not shown). One mitigating factor here is that a portion of the Gcn− phenotype of the ACT1/act1Δ strain is independent of YIH1 and thus may not involve impaired activation of GCN2. Considering that actin is a component of several transcriptional coactivator complexes (37), a defect in transcriptional activation by GCN4 could be responsible for the YIH1-independent component of the Gcn− phenotype of the ACT1/act1Δ strain. However, another intriguing possibility is that YIH1-mediated inhibition of GCN2 could be a localized phenomenon, restricted to the site of bud emergence, where an optimal level of eIF2 activity and translation initiation would support rapid bud growth. This model could explain why we failed to detect an increase in the total cytoplasmic pool of eIF2α-P in yih1Δ cells. There are precedents for localized regulation of eIF2α phosphorylation and translational control in mammalian cells (38, 39).

This last model assumes that the concentration of YIH1-actin complexes would be locally reduced at the site of bud growth. Most of the filamentous actin in yeast cells occurs in the cortical actin patches located preferentially near regions of exocytosis in the growing bud. There is increasing evidence that actin patches are sites of “compensatory” endocytosis needed to recycle plasma membrane and the protein secretion machinery utilized in exocytosis during bud emergence. The actin patches are highly dynamic structures, containing many actin-binding proteins and regulatory factors, and are constantly turning over (40). It has been predicted that an actin cable assembly machine also would be located near the sites of exocytosis in the bud to anchor the growing end of actin cables as they project outward into the mother cell to support polarized vesicle transport (41). It seems possible that the high concentration of other G-actin binding proteins involved in actin polymerization at the cortical patches and at the growing ends of actin cables would displace YIH1 from G-actin. This would enable localized YIH1-GCN1 complex formation and inhibition of GCN2 activity and provide a mechanism for polarized up-regulation of protein synthesis in the buds.

Acknowledgments—We thank Alex Vassilev for assistance with the Superdex200 column, David Botstein for actin antibodies, Thomas Dever for eIF2α antibodies, Matt Martos, Graham Favit and Hongfang Qiu for plasmids, Dhruba Chattoraj for supplying the light microscope, Beatriz Castillo for comments on the manuscript, and Felecia Johnson for help in preparing the manuscript.

REFERENCES
1. Chen, J. -J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 503–529, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Kaufman, R. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 529–546, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Ron, D., and Harding, H. P. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 547–560, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
4. Hinnebusch, A. G. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 185–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
5. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Mol. Cell 6, 1099–1108
YIH1 Is an Actin-binding Protein That Inhibits Protein Kinase GCN2 and Impairs General Amino Acid Control When Overexpressed

Evelyn Sattlegger, Mark J. Swanson, Emily A. Ashcraft, Jennifer L. Jennings, Richard A. Fekete, Andrew J. Link and Alan G. Hinnebusch

J. Biol. Chem. 2004, 279:29952-29962.
doi: 10.1074/jbc.M404009200 originally published online May 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404009200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 22 of which can be accessed free at http://www.jbc.org/content/279/29/29952.full.html#ref-list-1