Budding Yeast GCN1 Binds the GI Domain to Activate the eIF2α Kinase GCN2*

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When starved for a single amino acid, the budding yeast Saccharomyces cerevisiae activates the eukaryotic initiation factor 2α (eIF2α) kinase GCN2 in a GCN1-dependent manner. Phosphorylated eIF2α inhibits general translation but selectively derepresses the synthesis of the transcription factor GCN4, which leads to coordinated induction of genes involved in biosynthesis of various amino acids, a phenomenon called general control response. We recently demonstrated that this response requires binding of GCN1 to the GI domain occurring at the N terminus of GCN2 (Kubota, H., Sakaki, Y., and Ito, T. (2000) J. Biol. Chem. 275, 20243–20246). Here we provide the first evidence for the involvement of GCN1-GCN2 interaction in activation of GCN2 per se. We identified a C-terminal segment of GCN1 sufficient to bind the GI domain and used a novel dual bait two-hybrid method to identify mutations rendering GCN1 incapable of interacting with GCN2. The yeast bearing such an allele, gcn1-F229IL, fails to display derepression of GCN4 translation and hence general control response, as does a GI domain mutant, gcn2-Y74A, defective in association with GCN1. Furthermore, we demonstrated that phosphorylation of eIF2α is impaired in both mutants. Since GCN2 is the sole eIF2α kinase in yeast, these findings indicate a critical role of GCN1-GCN2 interaction in activation of the kinase in vivo.

Protein synthesis in eukaryotic cells is suppressed by stress-induced phosphorylation of eukaryotic initiation factor 2α (eIF2α)1 on a serine residue at position 51 (1). The phosphorylation converts eIF2α from the substrate to an inhibitor of eIF2B, the guanine nucleotide exchange factor of eIF2; phosphorylated eIF2α-GDP forms a stable complex with eIF2B to hamper recycling of eIF2-GDP to eIF2-GTP (2). Scarcity of eIF2-GTP accordingly decreases the level of the ternary complex composed of eIF2, GTP, and the charged initiator tRNA, a prerequisite for translational initiation, and hence leads to general suppression of protein synthesis. Thus, eIF2α kinases play pivotal roles in this famous translational control.

Mammalian cells have four eIF2α kinases, each of which is activated in response to a distinct stress. Heme-regulated inhibitor is activated by heme deprivation (3); double-stranded RNA-dependent kinase is activated by double-stranded RNA (3); RNA-dependent kinase-like endoplasmic reticulum kinase is activated by unfolded proteins (4, 5); and GCN2 is activated by serum or amino acid starvation (6, 7). In contrast, the budding yeast Saccharomyces cerevisiae has the sole eIF2α kinase, GCN2, the founding member of this family. The yeast GCN2 is activated by starvation for amino acids, glucose deprivation, purine limitation, and impaired tRNA synthetase activity (8–10). The gene for this kinase was originally identified in the studies of a response to amino acid starvation called general control of amino acid synthesis, and hence was termed GCN2 (general control nonderepressible 2).

The molecular mechanism underlying general control response is currently considered as follows. When the budding yeast starves for a single particular amino acid, free tRNAs, which are not charged with amino acids, accumulate within the cells and bind to a bipartite domain composed of the histidyl tRNA synthetase-related domain and the C-terminal ribosome-binding domain of GCN2 (11). This bipartite domain forms an inhibitory interaction with the kinase domain, which is disrupted upon binding of tRNAs (11). In addition to uncharged tRNAs, which unmask the kinase domain, genetic evidence suggests that in vivo activation of GCN2 requires another gene, GCN1, encoding a protein bearing a region homologous to translation elongation factor 3 (12). GCN1 forms a stable complex with the ATP-binding cassette protein GCN20 and functions on an elongating ribosome (13, 14). GCN2 is activated by uncharged tRNAs in the presence of GCN1 and phosphorylates eIF2α to suppress protein synthesis via the mechanism described above. However, the mRNA encoding GCN4 is selectively translated by a unique mechanism, which depends on the four short open reading frames (ORFs) preceding the one for GCN4 (10). The transcription factor GCN4 induces the expression of genes involved in various amino acid synthetic pathways.

In contrast to the action of uncharged tRNAs and the mechanism for derepressed translation of GCN4 mRNA, how GCN1 participates in the activation of GCN2 was poorly understood at the molecular level. Recently, we and others showed that a direct interaction between GCN1 and GCN2 is necessary for general control response, thereby providing the first insight into the underlying mechanism (15, 16).

In this study, we determine the minimal essential regions of GCN1 and GCN2 for the complex formation and demonstrate that phosphorylation of eIF2α, translational derepression of GCN4 mRNA, and general control response are impaired in the gcn1 and gcn2 mutants defective in this interaction. These
results provide the first direct evidence for a crucial role of GCN1-GCN2 interaction in the activation of the eIF2α kinase.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—The strains used in this study are summarized in Table I. Two-hybrid Assay and Other General Yeast Methods—The two-hybrid vectors, pGBK and pGAD424g, were described previously (17, 18). For high efficiency transformation, the protocol of Gietz and Schiestl (19) was adopted except for the addition of 10% dimethyl sulfoxide prior to the heat shock step (20).

**PCR-based Random Mutagenesis of GCN1**—A GCN1 DNA fragment (nucleotides 6142–7146) was cloned between the segments encoding the GAL4 activation domain (AD) and the C-terminal 7- amino acid (aa) region of CDC24, which bears a PCF motif and is called the PCF motif-containing region (PCCR) (18). We subjected the GAL4-AD-GCN1-PCCR fragment to error-prone PCR in 50 μl of 1× PCR buffer containing 1 unit of Taq DNA polymerase, 200 μM dATP, 2 μM dCTP, 2 μM dGTP, 2 μM dTTP, 2 μM MgCl2, 5 pmol of each primer under the following thermal cycling: 94 °C for 3 min, followed by 30 cycles of 95 °C for 15 s, 72 °C for 2 min, and 72 °C for 1 min. The amplified products of error-prone PCR were cloned into pGAD424g by a transformation-associated recombination technique (21) using PJ69–4Δ (MATa) as a host. Transformants were then mated with MaVX (MATa) bearing pGBK-GCN2 and pHIL-BEM1-PB1. The former plasmid encodes a hybrid protein between the GAL4 DNA-binding domain and GI domain of GCN2, and the latter one encodes a protein between LexA and the PB1 domain of BEM1 (aa 472–551), which specifically binds to PCCR of CDC24.2 Diploid cells were then plated onto synthetic complete medium (20) lacking Trp, Leu, and His (SC 2 Leu 2 Trp 2 His) supplemented with 20 mM 3-aminotriazole (3AT) and 0.2% 5-fluoro-orotic acid (5FOA).

**Immunoblotting Analysis of eIF2α Phosphorylation**—The yeast JBY2, JBY3, JBZ2, and JBZ3 cells were grown to midlogarithmic phase and shifted to YPAD (1% yeast extract, 2% peptone, 0.004% adenine sulfate, 2% glucose) or SD medium supplemented with 10 or 20 mM 3AT. Following incubation at 30 °C for 4 h, the cells were collected, resuspended in 1.0 ml of distilled H2O, and broken by the addition of 150 μl of breaking buffer (2 mM NaOH, 2 mM β-mercaptoethanol). Following incubation on ice for 10 min, the lysate was neutralized by the addition of 130 μl of 0.6% trichloroacetic acid. Proteins were then collected by centrifugation at 15,000 × g and washed twice with 700 μl of acetone. Protein extract equivalent to 106 cells was subjected to each lane of SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Filters were blocked by PBS-T (137 mM NaCl, 8.1 mM Na2HPO4, 2.68 mM KCl, 1.47 mM KH2PO4, 0.1% Tween 20, 4% nonfat dry milk). Phosphorylated eIF2α was visualized by using PBS-T containing an antibody that specifically recognizes eIF2α phosphorylated at serine 51 (BIO-SOURCE).

**RESULTS**

**The GI Domain Is the Minimal Essential Region to Interact with GCN1**—We previously showed that the characteristic GI domain (aa 1–125) occurring at the N-terminal extremity of GCN2 directly binds to GCN1 (15). Others also demonstrated that GCN2 interacts with GCN1 via its N-terminal 272-aa region, which contains the GI domain followed by an acidic region (16). To delimit the minimal essential region to interact with GCN1, we prepared a series of truncated mutants for the N-terminal region of GCN2 and tested them for binding with GCN1 using the yeast two-hybrid system.

All of the mutants bearing intact GI domain (aa 1–125) showed two-hybrid interactions with GCN1 (Fig. 1). The yeast cells with the longest GCN2 hybrid protein (aa 1–598), which includes the GI domain, acidic region, and degenerate protein kinase domain (αPR), showed higher β-galactosidase activity than those lacking αPK (aa 1–272). Although many factors affecting two-hybrid interactions, including intracellular level of hybrid proteins and efficiency of nuclear transport, make it difficult to evaluate strength of binding by this method, the result described above is in accordance with the one obtained using an in vitro pull-down binding assay (16). Notably, removal of the acidic region did not diminish β-galactosidase activity any more, and we failed to detect any evidence for this region to bind GCN1 (Fig. 1). In contrast, further deletion from either end of the GI domain (aa 1–125) completely abolished the interaction (Fig. 1). We had also shown that substitution of conserved residues in the GI domain abrogated the interaction

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2 T. Ito and H. Sumimoto, unpublished observation.
cated two-hybrid plasmids were exam-
ined for interaction with GCN1 using the yeast two-hybrid system. The yeast PJ69–4A for interaction with GCN1 using the yeast two-hybrid system. The yeast PJ69–4A cells (37) co-transformed with the indicated two-hybrid plasmids were examined for adenine- and histidine-independent growth and for the induction of β-galactosidase activity (in units (U)) driven by the lacZ reporter gene.

(15). These results indicated that the GI domain itself serves as the minimal essential region for the binding to GCN1 and that the acidic region has little if any role in the interaction.

Mapping the GCN2-binding Segment of GCN1—Two-hybrid screening using the GI domain as bait had revealed the C-terminal region around this region using PCR and tested their binding to the GI domain using the yeast two-hybrid system (Fig. 2). Two-hybrid interactions were examined for growth on the medium lacking adenine and histidine. The segment spanning aa 2064–2382 of GCN1 supported the growth as efficiently as those by longer constructs when expressed as a DNA-binding domain fusion. However, we failed to detect the interaction in the opposite orientation for unknown reason. Further deletion either from its N- or C-terminal end completely abolished the interaction (Fig. 2). From these results, we concluded that the region spanning amino acids 2064–2382 is sufficient for binding to the GI domain of GCN2.

Isolation of gcn1 Mutants Defective in Interaction with GCN2—We next intended to isolate gcn1 mutants defective in association with GCN2, because they would highlight critical residues for the recognition of the GI domain and because they can be used to examine the role of this interaction. For this purpose, we used a PCR-based random mutagenesis. However, since we had already pinpointed the minimal essential GCN1 region for GCN2 binding, we do not need nonsense mutations to truncate the protein anymore. To selectively obtain missense mutants, we developed a novel strategy described below (Fig. 3A).

We first modified the pGAD-GCN1-(2048–2382) plasmid so that the GAL4 AD-GCN1 fusion protein is further tailed with the PCCR of CDC24 (18), which specifically interacts with the PB1 domain occurring at the C-terminal end of BEM1.2 Following random mutagenesis to GCN1 by error-prone PCR, clones were selected for two-hybrid interaction with the PB1 domain, which guarantees that the hybrid protein retains the C-terminal PCCR and hence is not truncated within the GCN1 portion. From these untruncated populations, clones incapable of interacting with GCN2 were identified using the reverse two-hybrid selection based on URA3 reporter gene and 5FOA (25). Notably, this screening may be useful not only for the elimination of truncated proteins but also for the identification of those with missense mutations leading to protein instability, because such clones display weaker two-hybrid signals than the wild-type parental clone.

In practice, we used a dual bait two-hybrid system to perform both selections simultaneously. We prepared a mutagenized GAL4 AD-GCN1-PCCR library in Pj69–4A (MATα). These cells were then mated with Mavχ (MATα) cells that bear two

![FIG. 1. The GI domain of GCN2 is sufficient to interact with GCN1.](image)

![FIG. 2. Pinpointing the minimal essential segment of GCN1 to interact with the GI domain of GCN2.](image)
Role of Interaction between GCN1 and GCN2

GCN2 defective in interaction with GCN1 (15). Taken together, these results indicate that the interaction between GCN1 and GCN2 is necessary for general control of amino acid synthesis.

Mutants with Defective GCN1-GCN2 Interaction Fail to De-repress Translation of GCN4 mRNA—The GCn° phenotype described above suggests that the derepression of GCN4 translation is impaired in both gcn1-F2291L and gcn2-Y74A mutants. We thus examined the translation of GCN4 mRNA using a reporter construct bearing lacZ preceded by the characteristic GCN4 leader region, which is responsible for the derepression (23). The wild-type cells showed a remarkable induction of β-galactosidase activity under starved or derepressed conditions (Fig. 6). In contrast, the induction was severely impaired in both gcn1-F2291L and gcn2-Y74A cells (Fig. 6). Thus, the interaction between GCN1 and GCN2 is required for efficient derepression of GCN4 translation under amino acid-starved conditions.

Phosphorylation of eIF2α Is Impaired in Mutants with Defective GCN1-GCN2 Interaction—Finally, we intended to determine whether the eIF2α kinase is activated in these mutants upon amino acid starvation, because GCN2-independent mechanisms to derepress GCN4 translation are also possible (9, 10, 26–28). The phosphorylated eIF2α in mutants and their parental strains were examined under rich or poor conditions using an antibody specific to eIF2α phosphorylated at Ser-51. While phosphorylated eIF2α was barely detected under rich or repressed conditions, substantial phosphorylation of eIF2α was readily observed in the wild-type cells subjected to amino acid starvation (Fig. 7). In contrast, the induction of the phosphorylation was substantially impaired in the cells bearing gcn1-F2291L or gcn2-Y74A compared with their respective parental strains, although residual levels of phosphorylation were detected (Fig. 7). Since GCN2 is the sole eIF2α kinase in the budding yeast, these results indicate that the interaction with GCN1 is necessary for the GCN2 to be fully activated in amino acid-starved cells.

Discussion

GCN1 is required for the activation of GCN2 in the budding yeast under amino acid starvation; deletion or mutations of GCN1 were reported to abolish phosphorylation of eIF2α, which is mediated by the sole eIF2α kinase GCN2 (12, 14). It had, however, remained totally unknown how GCN1 activates GCN2 until we and others provided evidence for their direct association and its requirement for a general control response (15, 16). In this study, we determined the minimal essential regions for GCN1-GCN2 association and demonstrated, for the first time, that the interaction is critical to the activation of GCN2 itself, which leads to the selective derepression of GCN4 translation and subsequent general control response.

The minimal essential region of GCN2 to interact with GCN1 was mapped to its N-terminal 125 residues (Fig. 1), which we had designated as the GI domain (15). The N-terminal 272 residues involving the GI domain followed by an acidic region were reported necessary for the interaction by others (16). However, our data shown here and in a previous report (15) clearly indicate that the GI domain per se, but not the acidic region, serves as the core for the binding. As pointed out previously (15), the GI domain is found in various proteins other than GCN2. They include Impact (a product of an evolutionarily conserved gene that is genetically imprinted in mice) (29–31), AO7 (a RING finger protein interacting with ubiquitin-conjugating enzymes) (32), ARA54 (a coactivator of androgen receptor) (33), YDR152W (a yeast hypothetical protein), YLR419W (a member of the DEAH-box RNA helicase family), and so forth. It remains to be elucidated whether these GI domains also function in protein binding.

Yeast Cells with gcn1-F2291L Fail to Show General Control Response—To examine the role of GCN1-GCN2 interaction, we intended to generate a yeast strain bearing GCN1 incapable of interacting with GCN2. For this purpose, we chose F2291L substitution, because it occurs within a cluster of identically conserved amino acid residues among GCN1 from various species (Fig. 4). We also tagged GCN1 in this mutant and its parental strain with the T7-epitope at their C-terminal ends to facilitate detection by anti-T7 antibody.

As shown in Fig. 5A, comparable amounts of GCN1 were detected in wild type and gcn1-F2291L cells, thereby demonstrating that the mutation does not destabilize the full-length protein in vivo. We then examined these cells for sensitivity to 3AT, which is an inhibitor of HIS3, a typical GCN4 target, and has been used as an indicator of general control response. The mutant cells displayed remarkably higher 3AT-sensitivity than the parental strain (Fig. 5B). A similar phenotype was reported for yeast cells bearing the gcn2-Y74A allele, which encodes GCN2 defective in interaction with GCN1 (15). Taken together, these results indicate that the interaction between GCN1 and GCN2 is necessary for general control of amino acid synthesis.

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We also found that amino acid residues 2064–2382 comprise the minimal essential region of GCN1 to recognize the GI domain of GCN2 (Fig. 2). In accordance with this result, the segment spanning residues 2052–2428 of GCN1 was recently reported responsible for binding to GCN2 (24). In contrast to GCN2, the primary sequence of the GI domain-binding region of GCN1 lacks any apparently characteristic feature (Fig. 4). We thus took a random mutagenesis approach to identify critical residues for the recognition of GI domain.

For this purpose, we developed a unique dual bait two-hybrid strategy, which allows one to selectively identify missense mutations leading to defective interaction (Fig. 3A). Furthermore, this strategy would be also useful to eliminate mutants encoding unstable proteins, which can be identified as clones with weaker interaction between the C-terminally attached domain and its binding partner (i.e. PCCR and PB1). Therefore, it

*Fig. 4. GI domain-binding region of GCN1 and mutations abolishing interaction. The amino acid sequence of the GI domain-binding region of GCN1 (aa 2048–2382) is aligned with the corresponding regions of its homologs from fission yeast (GenBank™ accession number CAA92385), Arabidopsis (AAD38254), nematode (AAF60721), fruit fly (AAF45332), and human (BAA13209). Residues identically conserved among at least four species are boxed in black. #, the positions of amino acid substitutions found in the three single-point mutants defective in interaction with GCN2, namely F2291L, S2304P, and L2353P. *, R2259A substitution, which was also reported to abrogate the binding (24). The letters above the budding yeast sequence represent amino acid substitutions found in the seven double mutants, namely L2303S, V2329D, F2291S, V2376A, K2317R, L2319P, S2304P, L2356S, F2291I, T2307N, F2281L, Q2294R, and F2299A, R2328D. Note that we did not determine which of the two substitutions in each mutant is responsible for defective interaction.

*Fig. 5. General control response of gen1-F2291L mutant defective in interaction with GCN2. A, T7-tagged GCN1 and GCN1(F2291L) were visualized by immunoblotting with an anti-T7 tag antibody. B, the yeast cells JBZ2 (GCN1-T7) and JBZ3 (gcn1-F2291L-T7) were spotted onto agar plates for SC−Ura (top) or SC−Ura−2His (bottom).

*Fig. 6. Derepression of GCN4 translation in mutants defective in GCN1-GCN2 interaction. A, the yeasts MB758–5B (GCN1) and JBZ1 (gen1-F2291L), each bearing the GCN4-lacZ reporter plasmid p180 (23), were cultured in the indicated medium, and β-galactosidase (β-gal) activities were measured (in units (U)). B, the yeasts JBY4 (GCN2-T7) and JBY5 (gen2-Y74A-T7), which had been generated from JBY2 and JBY3 (15) by popping out the integrated URA3 marker, were transformed with p180 and examined for the induction of β-galactosidase activities.
The reduction in amounts (10) or impaired base modification encoding the tRNA pseudouridine 5′-hydroxymethyltransferase (28). Support minimal activation of the kinase. Notably, GCN1 and impaired induction of GCN2 are anchored onto ribosome through their respective ribosome-binding domains (14, 24, 35, 36), presumably, in such close proximity that they can interact. It is thus conceivable that the two proteins occasionally take a configuration leading to activation of the kinase although the interaction is considerably impaired by the mutations. In this context, it is intriguing to note that overexpression of GCN2 can suppress the Gcn4-phenotype of gcn1-R2259A, also defective in binding to GCN2, but not that of gcn1-ΔD, which encodes GCN1 totally lacking the GCN2-binding region (24).

Based on these observations, we conclude that the G domain-mediated association of GCN2 to GCN1 is necessary for the full activation of GCN2 kinase in vivo upon amino acid starvation and hence for efficient derepression of GCN4 translation, leading to general control response.

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