GI Domain-mediated Association of the Eukaryotic Initiation Factor 2α Kinase GCN2 with Its Activator GCN1 Is Required for General Amino Acid Control in Budding Yeast*

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Hiroyuki Kubota‡¶, Yoshiyuki Sakaki‡, and Takashi Ito¶‡
From the ‡Division of Genome Biology, Cancer Research Institute, Kanazawa University, 1-1 Takaramachi, Kanazawa 920-0934 and the §Human Genome Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

In response to the starvation of a single amino acid, the budding yeast Saccharomyces cerevisiae activates numerous genes involved in various amino acid biosynthetic pathways, all of which are under the control of transcription factor GCN4. This general amino acid control response is based on de-repressed translation of GCN4 mRNA, which is induced by the activation of the eIF2α kinase, GCN2. Although it is known that in vivo activation of GCN2 requires GCN1, the mode of GCN1 action remains to be elucidated at the molecular level. Here, we show that GCN2 interacts with GCN1 via the GI domain, a novel protein-binding module that occurs at the N terminus of GCN2, which is activated by starvation of amino acids, purine limitation, and impaired tRNA synthetase activity (3, 4). The eIF2α kinase GCN2 is named after its requirement in general amino acid control phenomenon, in which the budding yeast deprived of a single amino acid induces the expression of genes in the biosynthetic pathways not only for the starved one but also for other amino acids (3, 4). The genes activated in this response are all under the regulation of the transcription factor GCN4, and the specific de-repression of its translation is the very basis of this response. A series of excellent experiments led to the proposal of a unique mechanism for this translational de-repression, which depends on both the characteristic four, short, open reading frames in the leader region of GCN4 mRNA and the scarcity of the eIF2GTP-charged initiator tRNA ternary complex induced by the GCN2-mediated process described above (4). By contrast, the mechanism for the activation of GCN1 itself remains largely elusive. Uncharged tRNAs are assumed to activate GCN2; the histidyl tRNA synthetase-related region of GCN2 has been shown to bind tRNAs and to be necessary for its activation (5–9). In addition to uncharged tRNAs, it is known that in vivo activation of GCN2 requires a protein called GCN1, which bears a region homologous to translation elongation factor 3, forms a complex with the ATP-binding cassette protein GCN20, and functions on elongating ribosomes (10–12). It is, however, totally unknown how GCN1 activates GCN2.

In this study, we demonstrate that GCN1 binds to a novel protein-binding module, the GI domain, at the N terminus of GCN2 and that the interaction is essential for general amino acid control, thereby providing the first step toward the elucidation of the molecular mechanism for the activation of GCN2 by GCN1.

EXPERIMENTAL PROCEDURES

Yeast Strains—The yeast strains used were SFY526 (Mata ura3–52 his3200 ade2–101 lys2–801 trpl–901 leu2–3, 112 gal4–542 gal80–538 URA3::GAL1-lacZ), PJ69-4A (Mata trpl–901 leu2–3, 112 ura3–52 his3200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ1), MB78-5A (Mata ura3Δ), and FY1679H (Mata ura3–52 leu2–3 trpl–Δ63). Two-hybrid Assay and Other General Yeast Methods—The two-hybrid vectors, pGBK and pGAD424g, were described previously (14, 15). For high efficiency transformation, the protocol of Gietz and Schiestl (16) was adopted except for the addition of 10% dimethyl sulfoxide prior to the heat shock step (17). The filter assay for β-galactosidase was performed as described (14, 15).

In Vitro Protein Overlay Binding Assay—The DNA fragment encoding the N terminus of GCN2, termed GCN2-N (aa 1–125), was cloned in pGEX-2T (Amersham Pharmacia Biotech) to express GST-GCN2-N, whereas GCN1 (aa 2048–2382) was expressed using the pMAL-c2g vector (14, 15) as an MBP fusion protein in Escherichia coli.
subsequently linearized with MunI. Immunoprecipitates were resuspended in 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and a mixture of protease inhibitors. The immune complexes were collected by bivalent with anti-T7 tag antibody in 1.0 ml of TNTS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and a mixture of protease inhibitors). The immune complexes were washed with protein A-Sepharose (Amersham Pharmacia Biotech) and washed twice with the same buffer and once with kinase buffer (20 mM Tris-HCl supplemented with 1% Triton X-100, 0.1% SDS, and a mixture of protease inhibitors). These plasmids were introduced into the yeast FY1679H cells, and the transformants were tested for successful targeted integration by diagnostic colony polymerase chain reaction. These clones were then selected for 5-fluoroorotic acid resistance and examined for the desired allele replacement to obtain the strain JBY1 (Mata ura3Δ gen2-Y74A).

For epitope tagging of GCN2, we first constructed a pUC-URA3 derivative bearing a DNA fragment encoding the 3’-end portion of GCN2 open reading frame with its flanking region (nucleotide 4492–5166) followed by Ashbya gossypii TEF2 terminator derived from the kanMX cassette (18). Following the insertion of a T7 tag-encoding sequence by an inverse polymerase chain reaction-mediated procedure so that the GCN2 is C-terminally T7-tagged, the plasmid was linearized and transformed into MB758-5B and JBY1 to obtain the strains JBY2 (Mata ura3Δ GCN2-T7::URA3) and JBY3 (Mata ura3Δ gen2-Y74A-T7::URA3), respectively. These strains were spotted onto agar plates of SC medium supplemented with 10 mM 3-aminotriazole (3AT). The transformants were tested for successful transformation by a pull-down binding assay (data not shown). From these results, we concluded that the GI domain of GCN2 and Impact as well as in other proteins of apparently unrelated origins (Fig. 1). Because we first recognized these regions in GCN2 and Impact, we designated them as the GI domain. GCN2 Binds to GCN1 via Its GI Domain—The presence of GI domain in GCN2 prompted us to examine whether it also interacts with GCN1, because genetic experiments indicated that GCN1 is an in vivo activator of GCN2 (10–12). We first performed the yeast two-hybrid assays, in which the GI domain of GCN2 was expressed as a GAL4 DNA binding domain fusion from a bait plasmid pGBK-GCN2-N, whereas the YIH1-binding region of GCN1 (aa 2048–2382) as a GAL4 activation domain fusion from a prey plasmid pGAD-GCN1. A specific two-hybrid interaction between GCN2-N and GCN1 was readily detected; the PJ69-4A and SFY526 transformants showed adenine-independent growth (Fig. 2A) and elevated β-galactosidase activity (Fig. 2B), respectively, only when they bore both pGBK-GCN2-N and pGAD-GCN1. It should be noted that a two-hybrid interaction does not always mean that the two proteins directly bind each other but is occasionally mediated by a third-party yeast protein. To exclude the latter possibility, we tested the interaction between GCN1 and GCN2 in vitro using respective recombinant proteins expressed in E. coli. We expressed GCN2-N and GCN1 (aa 2064–2382) as a GST fusion protein (GST-GCN2-N) and an MBP fusion protein (MBP-GCN1), respectively. The protein overlay assay using these proteins showed that MBP-GCN1 specifically binds to GST-GCN2-N (Fig. 2C). The interaction was detected also by an in vitro pull-down binding assay (data not shown). From these results, we concluded that the GI domain of GCN2 directly interacts with GCN1.

Mutations to Conserved Motifs in the GI Domain of GCN2 Abolish Its Binding to GCN1—GI domains share characteristic motif-like sequences (Fig. 1). We thus replaced the conserved residues of these motifs with other amino acids and tested these mutant proteins for binding to GCN1. As shown in Fig. 3A, all of the GI domain mutants failed to show two-hybrid screening. The YIH1-binding region of GCN1 was pinpointed to amino acid residues (aa) 2064–2382, the region that lacks any significant homology with other proteins (data not shown). The GCN1-binding region of YIH1 was mapped to the less conserved N-terminal portion (aa 1–125) but not to the C-terminal region, highly conserved among all members of this family (data not shown). Although no homology had been noted for this N-terminal region, we analyzed it using the PSI-BLAST program (22) to reveal a modest homology between YIH1 and Drosophila GCN2 (23, 24). Based on this alignment and further PSI-BLAST search, we found that three characteristic motif-like sequences connected by spacers of variable lengths are conserved among the homologs of GCN2 and Impact as well as in other proteins of apparently unrelated origins (Fig. 1). Because we first recognized these regions in GCN2 and Impact, we designated them as the GI domain.

RESULTS

A GCN1-binding Protein, YIH1, Shares a Conserved GI Domain with GCN2—We previously isolated the mouse imprinted gene Impact (19), which encodes an evolutionarily conserved protein of unknown function belonging to uncharacterized protein family 29 (20, 21). As an approach to revealing the function of this protein family, we analyzed its budding yeast homolog YCR059C, or yeast impact homolog 1 (YIH1), and identified GCN1 as its potential binding partner through a two-hybrid program (22) to reveal a modest homology between YIH1 and Drosophila GCN2 (23, 24). Based on this alignment and further PSI-BLAST search, we found that three characteristic motif-like sequences connected by spacers of variable lengths are conserved among the homologs of GCN2 and Impact as well as in other proteins of apparently unrelated origins (Fig. 1). Because we first recognized these regions in GCN2 and Impact, we designated them as the GI domain.

GCN2 Binds to GCN1 via Its GI Domain—The presence of GI domain in GCN2 prompted us to examine whether it also interacts with GCN1, because genetic experiments indicated that GCN1 is an in vivo activator of GCN2 (10–12). We first performed the yeast two-hybrid assays, in which the GI domain of GCN2 was expressed as a GAL4 DNA binding domain fusion from a bait plasmid pGBK-GCN2-N, whereas the YIH1-binding region of GCN1 (aa 2048–2382) as a GAL4 activation domain fusion from the prey plasmid pGAD-GCN1. A specific two-hybrid interaction between GCN2-N and GCN1 was readily detected; the PJ69-4A and SFY526 transformants showed adenine-independent growth (Fig. 2A) and elevated β-galactosidase activity (Fig. 2B), respectively, only when they bore both pGBK-GCN2-N and pGAD-GCN1.

It should be noted that a two-hybrid interaction does not always mean that the two proteins directly bind each other but is occasionally mediated by a third-party yeast protein. To exclude the latter possibility, we tested the interaction between GCN1 and GCN2 in vitro using respective recombinant proteins expressed in E. coli. We expressed GCN2-N and GCN1 (aa 2064–2382) as a GST fusion protein (GST-GCN2-N) and an MBP fusion protein (MBP-GCN1), respectively. The protein overlay assay using these proteins showed that MBP-GCN1 specifically binds to GST-GCN2-N (Fig. 2C). The interaction was detected also by an in vitro pull-down binding assay (data not shown). From these results, we concluded that the GI domain of GCN2 directly interacts with GCN1.

Mutations to Conserved Motifs in the GI Domain of GCN2 Abolish Its Binding to GCN1—GI domains share characteristic motif-like sequences (Fig. 1). We thus replaced the conserved residues of these motifs with other amino acids and tested these mutant proteins for binding to GCN1. As shown in Fig. 3A, all of the GI domain mutants failed to show two-hybrid interaction between GCN2-N and GCN1 was readily detected; the PJ69-4A and SFY526 transformants showed adenine-independent growth (Fig. 2A) and elevated β-galactosidase activity (Fig. 2B), respectively, only when they bore both pGBK-GCN2-N and pGAD-GCN1.
interactions with GCN1, suggesting that they cannot interact with GCN1 in vivo. It is possible, however, that the mutated GI domains are unstable and that the reduced levels of these proteins caused the failure to detect two-hybrid interactions accordingly. The mutant GI domains were thus examined for binding to GCN1 using the in vitro protein overlay assay to examine the interaction with the overlaid MBP-GCN1.

We also found that similar mutations to the GI domain of YIH1 abolish its binding to GCN1 (data not shown). Therefore, the conserved motifs are essential for both GI domains to interact with GCN1.

GI Domain-mediated Association of GCN2 with GCN1 Is Required for General Control Response—To know a biological role for the interaction between GCN1 and GCN2, we generated a yeast strain bearing a mutated allele, *gcn2-Y74A*, whose product is incapable of interacting with GCN1 (Fig. 3). The GCN2 proteins in the mutant and its parental strain were T7-tagged at their C-terminal ends to facilitate the detection by immunoprecipitation. As shown in Fig. 4A, we confirmed that comparable amounts of GCN2 proteins were present in both wild and mutant strains. We then tested these cells for sensitivity to 3AT, which is an inhibitor of HIS3, a typical target of GCN4, and hence has been used as an indicator of general control response. As shown in Fig. 4B, the *gcn2-Y74A* strain displayed remarkably higher 3AT sensitivity than its parental GCN2 strain. These results indicate that the GI domain-mediated association of GCN2 to GCN1 is necessary for general amino acid control.

To obtain further evidence for the importance of this interaction, we conducted a series of overexpression experiments, in which the GI domain of GCN2 or its target region on GCN1 was expressed under the control of GAL1 promoter. According to the scenario postulating a critical role for the interaction, each binding domain would compete with its endogenous cognate for the binding partner to induce a dominant-negative effect on general control response. Indeed, overexpression of these proteins conferred 3AT sensitivity on cells (Fig. 5). This effect was not due to the general toxicity of the overexpressed proteins, because no growth defects were observed in the absence of amino acid starvation (Fig. 5). Notably, all of the GI domain mutants defective in GCN1 binding (Fig. 3) failed to confer the phenotype (Fig. 5). These results thus provide further evidence in support of GCN1-GCN2 interaction playing a critical role in general amino acid control.

Because both YIH1 and GCN2 bind to the same region of GCN1, it seems likely that the former also affects general control response as much as the latter. When the expression of full-length YIH1 was induced using the GAL1 promoter, the cells displayed 3AT sensitivity like those cells overexpressing dominant-negative forms of GCN1 or GCN2 (Fig. 5). The phenotype was co-segregated with its N-terminal half containing the GI domain but not with the C-terminal portion lacking the domain (Fig. 5). These findings not only indicate the importance of the GCN1-GCN2 interaction but also may suggest the possibility that the GI domain protein YIH1 participates in the down-regulation of general control response.
Interaction with ubiquitin-conjugating enzymes (25), human GCN2, or GI domain protein YIH1. The yeast FY1679H cells were transformed with YEpLKG-GCN1 (aa 2048–2383), YEpLKG-GCN2-N, YEpLKG-GCN2-N (E18A), YEpLKG-GCN2-N (E18K), YEpLKG-GCN2-N (Y74A), YEpLKG-GCN2-N (Y74AP75A), YEpLKG-YIH1-F, YEpLKG-YIH1-N, or YEpLKG-YIH1-C. Each transformant was spotted onto agar plates of SC medium containing glucose or galactose and 0 or 10 mM 3AT.

**DISCUSSION**

In this study, we showed that the eIF2α kinase GCN2 directly binds to its activator GCN1 via a novel protein-binding module designated as the GI domain. Furthermore, we demonstrated that the GI domain mutant gcn2-Y74A defective in this interaction fails to display general amino acid control. In this context, it is interesting to note that the gcn1 deleted for amino acid residues 2054–2426, which spans the pinpointed GCN2-binding region (aa 2064–2383), failed to restore growth of gcn1Δ cells on medium containing 3AT (12). We also showed that the overexpression of the GI domain of GCN2 or its target site on GCN1 impairs general control response. These results indicate that the GI domain-mediated association of GCN2 to GCN1 is required for general amino acid control response, thereby providing the first insight into the molecular mechanism for the activation of GCN2 by GCN1. It remains elusive whether this interaction is constitutive or induced on starvation.

Additionally, we found that the overexpression of the GI domain protein YIH1 can inhibit general control response, presumably by competing with GCN2 for the binding site on GCN1. This protein may thus play a role in the down-regulation of general control response. It would be intriguing to examine the deletion of this gene for general control by putting particular emphasis on its down-regulation, for instance, in response to replenishment of starved amino acids.

Finally, we assume that the GI domain represents a novel protein-binding module used in a variety of proteins. Indeed, in addition to homologs of GCN2 and Impact, the domain is found in other proteins including mouse AO7 (a RING finger protein interacting with ubiquitin-conjugating enzymes) (25), human ARA54 (a coactivator for androgen receptor) (26), and the budding yeast hypothetical proteins YDR152W and YLR419W. It is of particular interest to examine whether these GI domains also function in interactions with other proteins. Identification of their binding partners would also help to reveal structural features shared by the target regions of GI domains. Such efforts are currently under way.

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**Note Added in Proof**—A recent study also reported that the association of GCN1 with the N-terminus of GCN2 is required for GCN2 activation (Garcia-Barrio, M., Dong, J., Ufano, S., and Hinnebusch, A. G. (2000) *EMBO J.* 19, 1887–1899).

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