Ribosome-binding Domain of Eukaryotic Initiation Factor-2 Kinase GCN2 Facilitates Translation Control*

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A family of protein kinases regulate translation initiation in response to cellular stresses by phosphorylation of eukaryotic initiation factor-2 (eIF-2). One family member from yeast, GCN2, contains a region homologous to histidyl-tRNA synthetase juxtaposed to the kinase catalytic domain. It is thought that uncharged tRNA accumulating during amino acid starvation binds to the synthetase-related sequences and stimulates phosphorylation of the α subunit of eIF-2. In this report, we define another domain in GCN2 that functions to target the kinase to ribosomes. A truncated version of GCN2 containing only amino acid residues 1467 to 1590 can independently associate with the translational machinery. Interestingly, this region of GCN2 shares sequence similarities with the core of the double-stranded RNA-binding domain (DRBD). Substitutions of the lysine residues conserved among DRBD sequences block association of GCN2 with ribosomes and impaired the ability of the kinase to stimulate translational control in response to amino acid limitation. Additionally, as found for other DRBD sequences, recombinant protein containing GCN2 residues 1467–1590 can bind double-stranded RNA in vitro, suggesting that interaction with rRNA mediates ribosome targeting. These results indicate that appropriate ribosome localization of the kinase is an obligate step in the mechanism leading to translational control by GCN2.

Targeting of proteins to different compartments in the cell is an important mechanism regulating protein function. Proteins can associate with organelles, membranes, or components in the soluble fraction of the cell, providing proteins access to substrates or regulatory ligands. GCN2 is a member of a family of protein kinases that regulate translation by phosphorylation of eukaryotic translation initiation factor-2 (eIF-2)1 (1–4). Localization of GCN2 protein kinase to ribosomes appears to be a critical step leading to phosphorylation of eIF-2 in response to cellular stress.

Phosphorylation of eIF-2 is a well characterized mechanism regulating eukaryotic protein synthesis. The eIF-2 is a three-subunit protein that couples with Met-tRNA Met and participates in ribosomal selection of the start codon (5). During this initiation process, GTP bound to eIF-2 is hydrolyzed to GDP. Phosphorylation of the α subunit of eIF-2 at serine 51 impedes recycling of eIF-2-GDP to the active form, eIF-2-GTP. Currently, three protein kinases that phosphorylate this regulated site of eIF-2 have been characterized and their cDNAs cloned (1–3). Two of the proteins regulate protein synthesis in mammalian cells. The RNA-dependent protein kinase, PKR, participates in the antiviral defense mechanism mediated by interferon (6) and is also thought to function as a suppressor of cell proliferation and tumorigenesis (7–9), and the heme-regulated inhibitor kinase, HRI, is expressed predominately in reticulocytes and bone marrow and couples the synthesis of globin, the principal translation product in these tissues, to heme availability (10). The third eIF-2 kinase, GCN2, functions in the general amino acid control pathway of yeast Saccharomyces cerevisiae. In response to starvation for any one of several different amino acids, GCN2 phosphorylation of eIF-2 stimulates the translation of GCN4 (2, 4, 11, 12). The GCN4 protein is a transcriptional activator of more than 30 genes involved in amino acid biosynthesis.

This report centers on the regulation of the GCN2 protein kinase. The kinase catalytic domain of GCN2 shares sequence and structural similarities with the PKR and HRI that are distinguishable from other eukaryotic protein kinases (2, 13, 14). Adjacent to the kinase catalytic domain, GCN2 contains a region homologous to histidyl-tRNA synthetase (HisRS) that binds uncharged tRNA (12, 15). It is proposed that different uncharged tRNAs, which accumulate during amino acid starvation conditions, can interact with the synthetase-related domain of GCN2, resulting in activation of the kinase and phosphorylation of eIF-2 (2, 4, 12, 15, 16).

Another domain that is important for regulation of GCN2 involves targeting of the kinase to ribosomes. Ramirez et al. (17) showed by several criteria that GCN2 was associated with ribosomes. GCN2 co-migrated with free 40 S and 60 S ribosomal subunits, 80 S particles, and polysomes separated by sucrose gradient centrifugation. When ribosomes were dissociated into 40 S and 60 S subunits by omitting MgCl2 from the extract preparation, GCN2 remained associated with 60 S ribosomal subunits (17). GCN2 was also complexed with ribosomal subunits after electrophoresis in a composite agarose-acrylamide gel under non-denaturing conditions. The related eIF-2 kinase, PKR, was also found to interact with the ribosomal machinery based on biochemical fractionation (13, 18–20) and immunofluorescent staining (21). Two regions in the amino terminus of PKR, designated dsRNA-binding domains (DRBDs), contain several basic amino acids in a predicted a-helical structure that are related to a family of RNA-binding proteins (22–25). In addition to regulating kinase activity by dsRNA, the DRBD sequences facilitate PKR association with ribosomes (20). PKR targeting to ribosomes is proposed to enhance in vivo phosphorylation of eIF-2 by providing the kinase access to this substrate.

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1 The abbreviations used are: eIF-2, eukaryotic initiation factor-2; ds, double-stranded; DRBD, double-stranded RNA-binding domain; PAGE, polyacrylamide gel electrophoresis.
GCN2 Ribosome Binding Facilitates Translation Control

In this report, we describe the finding that GCN2 residues 1467–1590 can bind independently to ribosomes. This GCN2 domain contains a lysine-rich sequence with features similar to the core of the DRBDs. Alteration of these conserved lysine residues blocked both GCN2 interaction with ribosomes and stimulation of GCN4 expression in response to starvation for amino acids. As previously observed for the DRBDs, the lysine residues are essential for binding to dsRNA in vitro, suggesting that interaction with RNA mediates ribosome targeting. This cellular localization may provide GCN2 access to its eIF-2 substrate or to regulatory ligands. Furthermore, these findings indicate that related RNA-binding sequences facilitates the in vivo activities of both GCN2 and PKR in response to cellular stresses.

MATERIALS AND METHODS

Yeast strains and plasmid constructions—Yeast strains H1149 (MATa leu2-3,112 his4-lacZ) (15) and H1816 (MATa ura3-52 leu2-3,112 Δgcn2Δgcn2 LEU2 GN4-lacZ p1097 [SU2, LEU2]) (11) were transformed with different alleles of GCN2 in the following low copy number URA3-based plasmids: GCN2 in pZ22 (26); gcn2-m2 in pZ29 (12); GCN2 in pC102-2 (27); and p650 (15) is a derivative of pC102-2 containing a unique SacI restriction site introduced into GCN2 after the codon for residue 1467. There were no detectable differences in the GCN2 phenotype between strains transformed with plasmids pZ22, pC102-2, or p650. Plasmid p630 (26) contains GCN2 inserted into the high copy number URA3 plasmid YE2p2. Plasmids pS23 (12) and p644 (28) are derivatives of p630 containing the gcn2-m2 and gcn2-K559R alleles, respectively. To construct the gcn2-605 mutation, three lysine residues in the carboxyl terminus of GCN2, at positions 1483, 1484, and 1487, were altered by polymerase chain reaction at CTG, ATA, and ATA encoding leucine, isoleucine, and iso-leucine, respectively. The gcn2-605 mutation was introduced into plasmid p650 to generate the low copy number plasmid pS2-6, and into high copy number plasmid p630 to generate pS2-15.

To express the carboxyl-terminal portion of GCN2 in Escherichia coli we inserted a 0.7-kilo-base SacI to SalI restriction fragment from p650 into a pET-15b derivative. The resulting plasmid, pS2-3, encodes a polyhistidine amino-terminal sequence fused with GCN2 residues 1467–1590 downstream from the bacteriophage T7 promoter. Plasmid pS2-5 is a similar construct that encodes the gcn2-605 mutations. To express this histidine-tagged portion of GCN2 in yeast strain H1149 (MATa leu2-3,112 his4-lacZ) (28) or H1816 (MATa Δgcn2 ura3-52 leu2-3,112 trp1 his4-lacZ) (28) or H1819 (MATa Δgcn2 ura3-52 leu2-3,112 trp1 his4-lacZ) (28), polymerase chain reaction was used to insert the fusion gene into pYCDE2 (29). The resulting plasmid, pYCDE2, was transformed into pET-15b. Beads were washed three times in binding buffer and 400 μM imidazole in solution A. The molecular weight of the gcn2-1467–1590 fusion protein was 16,000, in agreement with that predicted from the DNA sequence. The protein was absent from an identically prepared extract from BL21(DE3) transformed with vector pET-15b. Additionally, both GCN2 and gcn2-605 recombinant proteins were recognized by polyclonal antiserum prepared against the carboxyl terminus of GCN2.

dsRNA binding assay—To measure binding of the gcns2-1467–1590 recombinant to dsRNA, we followed a procedure similar to that described by O’Malley et al. (32). Briefly, 5 μg of GCN2 or gcn2-605 recombinant protein were mixed with polyI:-polyC (bound to Sepharose 4B (Pharmacia Biotech) or Sepharose 4B alone in a 100-μl solution of binding buffer (20 μM HEPES, pH 7.5, 200 mM KCl, 10 mM MgCl2, and 0.5% Nonidet P-40). After incubating the binding mixtures for 15 min at room temperature, the beads were collected by brief centrifugation and resuspended in 10 × 300 μl solution A. Beads were resuspended three times in binding buffer and recombinant protein bound to the beads were eluted by adding the beads to 0.5% sodium deoxycholate instead of 0.5% SDS. Equal aliquots of each sample were separated by gel electrophoresis in a 15% SDS-polyacrylamide gel and the recombinant protein was visualized by staining the gel with Coomassie Blue.

Circular dichroism spectroscopy—CD spectra were measured on a Jasco J-720 spectropolarimeter. CD spectra were recorded with a 0.1-cm path length cuvette from 190 to 280 nm at a speed of 50 nm/min and with an increment of 1 nm. The mean residue ellipticities were calculated per residue and secondary structure contents were estimated using the reference spectra of Yang et al. (33) and the SSE-339 program (Japan Spectroscopic Co., Tokyo, Japan).

RESULTS

Carboxyl Terminal of GCN2 Functions as a Ribosome-binding Domain—GCN2 interaction with ribosomes is proposed to
facilitate stimulation of GCN4 translation in response to amino acid starvation. Ribosomal association appears to involve the carboxyl-terminal portion of GCN2 since deletion of this region reduced interaction of the kinase with ribosomes (17). To directly address whether the carboxyl terminus of GCN2 functions independently as a ribosome-binding domain, we expressed a polyhistidine fusion protein containing only GCN2 residues 1467–1590 in yeast strain WY294. Cell lysates were prepared as described under “Materials and Methods” and analyzed by sucrose gradient sedimentation. The distribution of the gcn2-1467–1590 in each gradient fraction was measured by immunoblot using polyclonal antiserum prepared against the carboxyl terminus of GCN2 (Fig. 1). The truncated GCN2 protein co-sedimented with ribosomes, with over 80% of gcn2-1467–1590 found in gradient fractions containing 60 S and 80 S particles and polysomes. Similar results were found when the truncated GCN2 protein was expressed in strain H1894 (Δgcn2) indicating that the ribosomal association was independent of endogenous full-length GCN2. By comparison, the GCN2 kinase catalytic sequences from 502 to 1054 expressed in yeast migrated free of ribosomes when analyzed in a similar sucrose gradient (Fig. 1B).

To further characterize the interaction of the carboxyl-terminal portion of GCN2 with ribosomes, we carried out sedimentation analysis in the absence of Mg\(^{2+}\), leading to the dissociation of ribosomes into free 40 S and 60 S particles. As previously observed for the full-length GCN2, we found that over 85% of the gcn2-1467–1590 protein co-migrated with free 60 S subunits (Fig. 1C). When these samples were treated with 0.5 M KCl, the gcn2-1467–1590 protein was dissociated from the 60 S subunit. This dissociation in the presence of KCl was described previously for full-length GCN2 (17), indicating that GCN2 is not an integral ribosomal protein. These results taken together indicate that the amino acid residues from 1467 to 1590 directly target GCN2 kinase to ribosomes.

**DRBD-related Sequences in the Carboxyl Terminus of GCN2 Are Required for Stimulation of the General Amino Acid Control Pathway**

Given that the DRBD sequences of PKR mediate association of this mammalian eIF-2 kinase with ribosomes (20), we examined whether there are sequence similarities between GCN2 residues 1467–1590 and the RNA-binding regions of PKR. Interestingly, residues 1479 to 1498 in GCN2 share sequence features similar to the core of the DRBD sequences found in PKR and other members of this RNA-binding family (Fig. 2). Although the structure of the RNA-binding regions in PKR have not yet been determined, the resolved structures of DRBD sequences from Staufen protein in *Drosophila melanogaster* and RNase III from *E. coli* show that this core region is α-helical (23, 34). The conserved lysine residues are located at the amino-terminal portion of the helix and are proposed to directly contact dsRNA (25, 35). Analysis of the GCN2 sequence also leads to a prediction of positively charged residues clustered in an α-helical secondary structure (Fig. 2).

during gradient analysis. In C and D, cycloheximide and MgCl\(_2\) were absent from the sucrose gradients, leading to 80 S ribosome dissociation into free 40 S and 60 S subunits. The sucrose gradient in D was supplemented with 0.5 M KCl to remove nonintegral ribosomal proteins. With the removal of nonintegral proteins, the free subunits migrated more slowly in the gradient and the arrows in D indicate the positions of 40 S and 60 S subunits in gradients analyzed in the absence of KCl. The top panels in each figure show the \(A_{254}\) profile of the gradient, with free 40 S and 60 S subunits, 80 S ribosomes, and polysomes indicated. The overlaid histogram shows the portion of gcn2-1467–1590 or gcn2-502–1054 protein found in each gradient fraction as measured by immunoblot analysis (bottom panels). Lane M in the immunoblot assay is the cellular lysate applied to the sucrose gradient. Sizes are indicated in kilodaltons to the left of each panel.
To determine the importance of this lysine-rich sequence of GCN2 in the stimulation of general control, we altered the three conserved lysine residues as shown in Fig. 2. The resulting mutant allele, termed gcn2-605, was introduced into the strain H1816 (Δgcn2 GCN4-LacZ) on either a low copy or high copy number plasmid. The level of GCN4-LacZ enzyme activity was 5-fold higher in the strain H1816 expressing wild-type GCN2 with different DRBD sequences. Amino acid residues with capital letters represent identities with proposed consensus sequences of the DRBDs (22–25). Numbers to the right of the sequences indicate the position of the last aligned residue in the indicated protein. Lysine residues at GCN2 positions 1483, 1484, and 1487 were altered to leucine, isoleucine, and isoleucine, respectively, in the gcn2-605 mutant allele. Dashes indicate a gap in the sequence. Bottom, helical wheel projection of GCN2 residues 1481 to 1498 predicted using the Garnier Plot program (54). Lysine residues in bold-capital letters are positions 1483, 1484, and 1487.

![Diagram of GCN2 protein](image)

**Fig. 2.** Carboxy-terminal sequence of GCN2 shares sequence features with the DRBDs. Top, the box designated GCN2 represents the 1,590-amino acid-long sequence of the GCN2 protein kinase. GCN2 contains domains with homology to protein kinases and histidyl-tRNA synthetases (HisRS) (15). In the amino-terminal portion of GCN2 is an additional domain with sequences related to subdomains Vb to XI of eukaryotic protein kinases that is required for kinase function in vivo and in vitro (16, 26). Middle, alignment of the carboxy terminus of GCN2 with different DRBD sequences. Amino acid residues with capital letters represent identities with proposed consensus sequences of the DRBDs (22–25). Numbers to the right of the sequences indicate the position of the last aligned residue in the indicated protein. Bottom, helical wheel projection of GCN2 residues 1481 to 1498 predicted using the Garnier Plot program (54). Lysine residues in bold-capital letters are positions 1483, 1484, and 1487.

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![Diagram of GCN2 protein](image)

**Fig. 3.** Immunoblot analysis of GCN2 protein. Protein extracts were prepared from strain H1149 (Δgcn2) transformed with different plasmid-borne GCN2 alleles as described under “Materials and Methods.” Equal amounts of protein extracts were separated by 7.5% SDS-PAGE, transferred to nitrocellulose paper and GCN2 protein was assayed using antiserum prepared against a TrpE-GCN2 fusion protein. Lanes are designated by wild-type GCN2 or mutant gcn2-605 protein expressed from low copy number (L.C.) or high copy number (H.C.) plasmids. The steady-state levels of gcn2-605 and GCN2 proteins expressed from low copy number plasmids differed by less than 20% as judged by densitometry. Comparison of high copy levels of GCN2 indicated that gcn2-605 protein is 60% of wild-type kinase. The lane designated Δgcn2 is strain H1149 transformed with vector YEp24.

![Diagram of GCN2 protein](image)

**TABLE I**

| Plasmid-borne allele | Enzyme activity (nmol/min/mg of protein) | Growth on 3-AT |
|----------------------|-----------------------------------------|---------------|
| Low copy GCN2        | 20 100 150 830                           | +             |
| Δgcn2                | 10 70 80                                 |               |
| Low copy gcn2-605    | 14 23 90 80                              | –             |
| High copy gcn2-605   | 21 23 140 121                            | –             |

**β-Galactosidase enzyme activity was assayed in extracts prepared from transformants of H1816 (Δgcn2 GCN4-LacZ) and H1149 (Δgcn2 HIS4-LacZ) containing the indicated GCN2 alleles.** R, repressed or non-starved growth conditions; D, derepressed growth conditions imposed by the addition of 3-AT to the culture medium. Each GCN2 allele was encoded on a URA3-based plasmid as follows: GCN2 encoded in pC102-2; Δgcn2 is vector YEp50; gcn2-605 encoded on low copy number plasmid PSZ-6 and high copy-number plasmid pSZ-15. Results shown are averages of three independent assays, and the individual measurements deviated from the average values shown here by 20% or less. Growth of H1149 transformants on 3-aminotriazole (3-AT) agar plates is a measure of stimulation of HIS3 expression in response to histidine limiting growth conditions. Symbols: +, confluent growth of replated patches of cells after 2 days at 30°C; nondiscernible growth under the same conditions.

The level of GCN4-LacZ enzyme activity was 5-fold higher in the strain H1816 expressing wild-type GCN2 than when these cells were grown under repressed or non-starved conditions (Table I). In the absence of GCN2 function there was no increase in GCN4-LacZ enzyme activity in response to histidine starvation. The gcn2-605 mutant strain also showed very little increase in GCN4-LacZ enzyme activity during amino acid limiting conditions, even when the gcn2-605 allele was expressed from a high copy number plasmid. Consistent with the idea that the gcn2-605 protein is impaired for stimulation of the general control pathway, gcn2-605 strains also failed to grow on agar medium supplemented with 3-aminotriazole and showed no increase in the expression of HIS4-lacZ, a gene transcriptionally activated by GCN4, during starvation conditions. This reduction in gcn2-605 stimulation of the general amino acid control pathway is not due to instability of the mutant protein, as an immunoblot assay revealed steady-state levels of gcn2-605 protein to be comparable to wild-type GCN2 (Fig. 3). In fact, even when the gcn2-605 protein encoded on the high copy number plasmid was elevated 20-fold compared with the low copy number trans-
domain was more sensitive than immunoblot assays and when compared in parallel experiments was found to be an accurate measure of steady-state protein levels. Consistent with this earlier study, we found wild-type GCN2 kinase associated with free 40 S, 60 S, and 80 S particles and polysomes. We observed a similar pattern of GCN2 distribution in the sucrose gradient when we fractionated a cellular extract prepared from yeast cells expressing the kinase from a high copy number plasmid and measured GCN2 protein by immunoblot analysis (data not shown).

To address whether the gcn2-605 mutant protein was altered for ribosomal association, a gcn2-605 strain lysate was fractionated by sucrose gradient sedimentation, followed by the immunoprecipitation kinase assay (Fig. 4). We observed that the autophosphorylation level of the mutant protein in the immunoprecipitation kinase assays was near that of wild-type GCN2 (Fig. 4, data not shown). The gcn2-605 protein was found near the top of the sucrose gradient, in fractions free of ribosomes. In a parallel experiment, lysate prepared from cells expressing the mutant kinase from a high copy number plasmid was analyzed by sucrose gradient centrifugation, followed by immunoblot analysis. Even when gcn2-605 protein levels were elevated in the cell we found the mutant kinase in the top portion of the gradient in fractions containing no ribosomes (data not shown).

Two additional mutant versions of GCN2 were next analyzed to determine whether the function of other domains of the kinase were essential for targeting to the translation machinery. First, we fractionated extracts prepared from cells expressing the mutant gcn2-K559R protein that contains a substitution of the conserved lysine in the ATP-binding sequence in the kinase catalytic domain, rendering it catalytically impaired (26). Immunoblot analysis of the gcn2-K559R protein in the gradient fractions revealed a similar profile to that determined for wild-type GCN2 (Fig. 4). Second, we analyzed a GCN2 mutant protein containing substitutions in the conserved Tyr and invariant Arg at positions 1050 and 1051, respectively, in the motif 2 sequence of the synthetase-related domain. Previously, this gcn2-m2 protein was shown to be blocked in its ability to stimulate GCN4 expression in response to amino acid limitation and was greatly reduced for binding in vitro to uncharged tRNA compared with wild-type GCN2 (12). Fractionation of the gcn2-m2 protein in the sucrose gradient revealed a ribosomal profile for the mutant protein similar to that determined for the wild-type GCN2 protein. Taken together with the ribosomal association of the kinase defective-gcn2-K559R protein, we conclude that binding of uncharged tRNA to the HisRS-related domain and invariant Arg at positions 1050 and 1051, respectively, in the motif 2 sequence of the synthetase-related domain. Previously, this gcn2-m2 protein was shown to be blocked in its ability to stimulate GCN4 expression in response to amino acid limitation and was greatly reduced for binding in vitro to uncharged tRNA compared with wild-type GCN2 (12). Fractionation of the gcn2-m2 protein in the sucrose gradient revealed a ribosomal profile for the mutant protein similar to that determined for the wild-type GCN2 protein. Taken together with the ribosomal association of the kinase defective-gcn2-K559R protein, we conclude that binding of uncharged tRNA to the HisRS-related domain and subsequent activation of the kinase catalytic activity is not a prerequisite for ribosomal association of GCN2.

The Carboxyl Terminus of GCN2 Can Bind dsRNA and Contains an α-Helical Structure—Given the well characterized affinity of the DRBD regions of PKR for dsRNA, we wanted to address directly whether the carboxyl-terminal domain of GCN2 shared this binding property. We overexpressed in E. coli a recombinant protein containing the GCN2 sequence from residues 1467 to 1590 fused to an amino-terminal sequence containing six contiguous histidine residues. Nickel chelation resin was used to purify the recombinant fusion protein, and in parallel, we overexpressed and purified a similar fusion protein containing the gcn2-605 residue substitutions. Both proteins were purified to apparent homogeneity as judged by Coomassie staining of an SDS-polyacylamide gel after electrophoretic analysis (Fig. 5). Purified GCN2 or gcn2-605 recombinant protein were mixed in a buffer solution containing poly(I)-poly(C)
indicating that the polyhistidine tag did not contribute to the spectrum was shown to be similar to that determined in Fig. 6, of the cleaved version of the recombinant protein, the CD move the tag from the recombinant protein. After purification the amino-terminal polyhistidine and GCN2 sequences to re-utilized a thrombin proteolytic cleavage site located between the polyhistidine tag contributed to the helical properties, we secondary structure of the GCN2 protein. To assess whether gcn2-605 recombinant protein showed a similar spectrum sug-192–193 nm. This spectrum is characteristic of helix-contain-lar dichroism spectrum of the recombinant GCN2 protein (Fig. 6). The spectrum contains a broad negative trough of ellipticity with minimum near 208 and 222 nm and positive ellipticity at 6). The spectrum contains a broad negative trough of ellipticity of this region of GCN2 with DRBD sequences that facilitate interaction similar to wild-type GCN2 (Fig. 4). GCN2 interaction with uncharged tRNA also does not appear to be a prereq-uise step leading to ribosome association, since the gcn2-m2 mutant protein, containing substitutions in the HisRS-related domain that greatly reduce binding of uncharged tRNA in vitro (12), co-fractionates with ribosomes in the sucrose gradient (Fig. 4). These results suggest that the DRBD-related sequence of GCN2 interacts with ribosomes independent of autopropho-lyation of GCN2 or stimulation of kinase activity by uncharged tRNA that accumulates during amino acid starvation conditions.

What role does association with ribosomes play in the process leading to GCN2 phosphorylation of eIF-2α? Targeting to ribosomes could provide GCN2 access to its substrate eIF-2. During initiation of translation, eIF-2 is associated with ribo-
somal subunits, providing ribosome-associated GCN2 proximity to its substrate (5, 17). A second possible role of ribosome association in the regulation of GCN2 kinase is that it provides a vehicle for GCN2 to monitor the levels of uncharged tRNA in the cell. GCN2 interaction with ribosomes may be adjacent to the aminoacyl (A) site, with the HisRS-related domain moni-toring uncharged tRNA that enters and is released from this site during the elongation of step in protein synthesis. The proximity of GCN2 in the ribosome would be similar to that of the RelA protein of E. coli. In this example, the ppGpp synthetase activity of RelA is thought to be stimulated by uncharged tRNA that binds the A site during amino acid starvation conditions (36). This model implies that the uncharged tRNA levels in the cell can be more efficiently monitored by GCN2 when the kinase is associated with ribosomes compared with the kinase being dispersed throughout the cytoplasmic solution.

In support of the idea that a ribosomal context facilitates monitoring of uncharged tRNA levels, Deutscher and col-leagues (37–39) proposed that there is cellular channeling pro-
cess for delivery and release of tRNA to the translation appara-tus. During this channeling, tRNAs are directly transferred from aminocyl-tRNA synthetases to the elongation factors to the ribosomes without being freely soluble in the cytoplasmic fluid. After deacylation during the translation process, tRNAs reassociate with their cognate aminocyl tRNA synthetases to repeat the cycle. Perhaps, during conditions of amino acid limitation, uncharged tRNAs whose levels are elevated, enter and are released from the A site with increased frequency by this channeling process. The ribosome localization of GCN2 would provide the kinase access to one of the channeling steps, allowing the synthetase-related domain of GCN2 to monitor the levels of uncharged tRNAs. Ancillary factors such as GCN1 and GCN20 (40, 41), that form a heterocomplex associated with ribosomes and are required for high levels of GCN2 phospho-rylation of eIF-2 during histidine limitation, may function to direct uncharged tRNAs from the A site to the HisRS-related domain. 

**DISCUSSION**

GCN2 is a multidomain protein kinase that regulates GCN4 translation initiation in response to amino acid starvation. In this report, we observed that the carboxyl terminus of GCN2 from residues 1467 to 1590 directly facilitate targeting of the kinase to the translation machinery. Interestingly, comparison of this region of GCN2 with DRBD sequences that facilitate ribosome interaction of a related eIF-2 kinase, PKR, revealed a lysine-rich sequence in GCN2 with features similar to the core of this RNA-binding domain. Substitutions of lysine residues conserved among DRBDs block association of GCN2 with ribosomes and impaired the ability of the kinase to stimulate the general control pathway in response to amino acid limitation. These results suggest that appropriate localization of GCN2 to the translational machinery is an obligate step in the mecha-nism leading to kinase phosphorylation of eIF-2.

**Role of Ribosome Binding in Stimulating GCN2 Phosphorylation of eIF-2α in Response to Amino Acid Starvation Conditions**—Three mutant alleles of GCN2 were examined for their effects on association of the kinase to ribosomes. While the gcn2-605 protein, containing substitutions in the DRBD-related sequence, was not associated with ribosomes, the kinase-defective gcn2-K559R protein showed a pattern of ribosome interaction similar to wild-type GCN2 (Fig. 4). GCN2 interaction with uncharged tRNA also does not appear to be a prereq-uise step leading to ribosome association, since the gcn2-m2 mutant protein, containing substitutions in the HisRS-related domain that greatly reduce binding of uncharged tRNA in vitro (12), co-fractionates with ribosomes in the sucrose gradient (Fig. 4). These results suggest that the DRBD-related sequence of GCN2 interacts with ribosomes independent of autopropho-lyation of GCN2 or stimulation of kinase activity by uncharged tRNA that accumulates during amino acid starvation conditions.
domain of GCN2 (42).

General Role of DRBD Sequences in Targeting Proteins to Ribosomes—Over 20 different proteins have been identified that contain DRBD sequences (24). Many of these proteins have been characterized only by genomic sequencing projects and the role of DRBDs in facilitating their physiological functions are currently unclear. We have shown that DRBD-containing protein, X1rbpa, that is the Xenopus homolog of TAR-RNA-binding protein, was also found to associate with ribosomes (43). Another likely example is the protein encoded by the S. cerevisiae gene, was also found to associate with ribosomes (43). An- 

The specific ribosomal locations that can accommodate by different DRBD sequences appear to be variable, with ribosomal dissociation experiments indicating that GCN2 and PKR are localized to 60 S and 40 S ribosomal subunits, respectively. These different ribosomal binding sites suggest that DRBD sequences bind to unique double-stranded regions in rRNA. Amino acid residue differences between the DRBDs would be expected to mediate this specificity for different RNA sequences and structures. Additionally, the fact that many proteins contain multiple DRBD sequences suggests that multiple RNA-binding elements may contribute to the affinity for unique ribosomal sites.

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