Polyribosome Binding by GCN1 Is Required for Full Activation of Eukaryotic Translation Initiation Factor 2α Kinase GCN2 during Amino Acid Starvation*

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The protein kinase GCN2 mediates translational control of gene expression in amino acid-starved cells by phosphorylating eukaryotic translation initiation factor 2α. In *Saccharomyces cerevisiae*, activation of GCN2 by uncharged tRNAs in starved cells requires its direct interaction with both the GCN1-GCN20 regulatory complex and ribosomes. GCN1 also interacts with ribosomes in cell extracts, but it was unknown whether this activity is crucial for its ability to stimulate GCN2 function in starved cells. We describe point mutations in two conserved, noncontiguous segments of GCN1 that lead to reduced polyribosome association by GCN1-GCN20 in living cells without reducing GCN1 expression or its interaction with GCN20. Mutating both segments simultaneously produced a greater reduction in polyribosome binding by GCN1-GCN20 and a stronger decrease in eukaryotic translation initiation factor 2α phosphorylation than did mutating in one segment alone. These findings provide strong evidence that ribosome binding by GCN1 is required for its role as a positive regulator of GCN2. A particular mutation in the GCN1 domain, related in sequence to translation elongation factor 3 (eEF3), decreased GCN2 activation much more than it reduced ribosome binding by GCN1. Hence, the eEF3-like domain appears to have an effector function in GCN2 activation. This conclusion supports the model that an eEF3-related activity of GCN1 influences occupancy of the ribosomal decoding site by uncharged tRNA in starved cells.

Phosphorylation of the α subunit of eukaryotic translation initiation factor 2α (eIF2α)1 provides a key mechanism for down-regulating protein synthesis in response to nutrient starvation or stress in mammalian and yeast cells (reviewed in Ref. 1). In its GTP-bound state, the heterotrimeric eIF2 complex delivers initiator methionyl-tRNA to the 40 S ribosome at an early stage of the initiation pathway. The eIF2 is ultimately released from the ribosome in an inactive GDP-bound state and must be recycled to eIF2-GTP by the guanine nucleotide exchange factor eIF2B for a new round of initiation to occur. Phosphorylation of eIF2α converts eIF2-GDP to an inhibitor of eIF2B, interfering with eIF2 recycling and decreasing the rate of translation initiation. In *Saccharomyces cerevisiae*, eIF2α phosphorylation also increases the translation of *GCN4* mRNA, encoding a transcriptional activator of amino acid biosynthetic enzymes subject to general amino acid control (GAAC). Thus, the rate of amino acid biosynthesis is stimulated by GCN4 in parallel with the reduction in general protein synthesis as a dual mechanism for replenishing amino acids in starved yeast cells. The diminished initiator methionyl-tRNA recruitment produced by eIF2α phosphorylation allows ribosomes to overcome the inhibitory function of four upstream open reading frames present in the *GCN4* mRNA leader and initiate at the *GCN4* start codon instead (reviewed in Ref. 1). In mammalian cells, expression of transcription factor ATF4 is stimulated at the translational level in response to eIF2α phosphorylation by a mechanism involving upstream open reading frames very similar to that elucidated for *GCN4* mRNA in yeast (2, 3). Mammals contain four eIF2α kinases (PKR, PERK, HRI, and GCN2) activated in response to different kinds of stress or starvation, whereas GCN2 is the sole member of this kinase subfamily in *S. cerevisiae*.

GCN2 is activated by uncharged tRNAs that accumulate in amino acid-starved cells and bind to a histidyl-tRNA synthetase (HisRS)-like domain located C-terminal to the kinase domain (1). We showed previously that GCN2 activation in starved cells additionally requires interaction of the GCN2 N-terminal domain with the GCN1-GCN20 regulatory complex through a C-terminal segment of GCN1 (residues 2052–2428 within area D shown in Fig. 1A) (4, 5). GCN1 is a large, 296-kDa protein containing a central domain with strong sequence similarity to the N-terminal portion of fungal translation elongation factor 3 (eEF3) (6). eEF3 promotes release of deacylated tRNAs from the ribosomal exit site and thereby stimulates delivery of charged tRNAs to the acceptor site (A-site) by eEF1A-GTP (7). The eEF3-like domain in GCN1 contains the binding domain for the N-terminal portion of GCN20 (8), whereas the remainder of GCN20 is related to the C terminus of eEF3, including the two ATP-binding cassettes (9). Thus, formation of the GCN1-GCN20 complex juxtaposes the domains in these two proteins that are related to different segments of eEF3 (Fig. 1).

GCN1 associates with elongating ribosomes (polyribosomes) in cell extracts, and this interaction is stimulated by ATP in a manner dependent on the ATP-binding cassettes in GCN20 (8). GCN2 also has ribosome binding activity that appears to be

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1 The abbreviations used are: eIF2α, eukaryotic translation initiation factor 2α; GAAC, general amino acid control; HisRS, histidyl-tRNA synthetase; eEF3, eukaryotic elongation factor 3; A-site, acceptor site; WCE, whole cell extract; 3AT, 3-amino-2,4-triazole; SC, synthetic complete.

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critical for its function in vivo (10, 11). GCN1 and GCN2 both bind to polyribosomes in cell extracts independent of the respective domains required for their interaction with another (5, 10, 11). Likewise, complex formation between GCN1 and GCN2 can occur in the absence of their ribosome binding domains (4, 5). Hence, it appears that GCN1 and GCN2 independently contact one another and the 80 S ribosome to promote assembly of a GCN1-GCN2 complex.

For construction of the low copy plasmid pES182-8 containing gen1-M7A, two PCR amplifications were performed using p2367 as template, primer pair ES900-17 and ES900-10, and primer pair ES900-11 and ES900-18. In a subsequent PCR reaction, the two PCR amplifications were fused together using primers ES900-7 and ES900-8. The Nhel-MluI-digested PCR fragment was ligated into the similarly digested plasmid p2367, yielding pES182-8.

The low copy plasmid pES257-12-2, containing gen1-M7D, was constructed similarly to plasmid pES182-8, using primers ES900-11 and ES900-12 instead of primers ES900-9 and ES900-10. For the construction of the low copy plasmid pES263-34-1, containing gen1-M1AM7A, the MluI-SalI fragment of pES182-8 was replaced by the MluI-SalI insert yielded from pES176-4.

**Polyribosome Analysis of Cell Extracts from Cross-linked Cells—**In vivo association of proteins with polyribosomes was determined as described previously with a few modifications (16). Briefly, yeast cells were grown to exponential phase in 300 ml of SC medium to an A600 of ~1 in 1-L baffled flasks. Cells were pelleted into a 400-ml centrifuge bottle containing 75 g of ice chips and 8.1 ml of formaldehyde (1% final concentration). Cells were then mixed and incubated on ice for 1 h with gentle mixing every 15 min. Cells were pelleted for 3 min at 4000 rpm (model J6 centrifuge, Beckman), washed with 6 ml of breaking buffer (20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 1 complete protease inhibitor tablet without EDTA (Roche Applied Science) per 25 ml, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 0.1 mg/ml each of pepstatin, aprotinin, and leupeptin), and resuspended in 400 μl of breaking buffer. Cells were broken with a volume of glass bead equivalent to the volume of packed cells by vortexing eight times for 30 s with 30 intervals on ice water. Cell debris was removed via centrifugation in a tabletop centrifuge (Eppendorf) for 10 min at 14,000 rpm, and 20 A600 units of extract were resolved on a 4.5–45% sucrose density gradient by centrifugation for 2 h at 39,000 rpm at 4 °C using the SW41Ti rotor (Beckman). 700-μl fractions were collected whereas scanning continuously for A260 units using the Brandel density gradient fractionator, model BR-184.

**Protein Techniques—**Proteins were separated by SDS-PAGE using gradient gels (4–12% Tris/glycine gels, Invitrogen; 4–20% criterion gels, Bio-Rad) and transferred to nitrocellulose membranes (Invitrogen or Bio-Rad) according to the manufacturer’s protocol. Proteins were detected by the enhanced chemiluminescence detection system (Amer sham Biosciences) using antibodies against GCN20 (CV1317, 1:2000 (9)), eIF2α (1:2000 (17)), eIF2γ phosphorylated on Ser-51 (1:5000, BioSource International, Inc.), actin (1:5000 (18)), RPL39 (1:5000, from Dr. Maurice Swanson), or c-myc (1:1000, Roche Applied Science). Immune complexes were visualized using horseradish peroxidase conjugated to donkey anti-rabbit antibodies, to sheep anti-mouse antibodies (for detection of c-myc antibodies), or to protein A (for detection of actin antibodies) (Amer sham Biosciences).

**RESULTS**

**Basic Residues in Area B of GCN1 Are Required for Optimal Polyribosome Binding in Vivo—**We showed previously that ribosome binding by GCN1 was impaired by deletions of areas A, B, or C in GCN1-GCN2 complex is crucial for its function in activation of RelA protein by uncharged tRNA in the transfer of uncharged tRNA from the A-site to the HeisRS-like domain in GCN2 for kinase activation (5, 8). This proposed mechanism resembles that demonstrated in Escherichia coli for activation of RelA protein by uncharged tRNA bound to the A-site, which mediates the stringent response to amino acid starvation in bacteria (13, 14).

We showed previously that deletions of regions A, B, or C in GCN1, encompassing the N-terminal 77% of the protein (see Fig. 1A), destroys GCN1 regulatory function and impairs ATP-dependent polyribosome binding by GCN1 in cell extracts (5). This result suggested that GCN1 has multiple points of contact with the ribosome. Although the deletions of regions A, B, or C did not destabilize the remainder of GCN1 protein or impair its interaction with GCN2, it is possible that these large deletions destroy another function of GCN1 other than ribosome binding. Hence, it was uncertain whether stable ribosome association by the GCN1-GCN2 complex is crucial for its function in activating GCN2. To validate this important aspect of our model, we set out to isolate point mutations in GCN1 that would impair polyribosome binding by GCN1 without affecting GCN1 expression or complex formation with GCN2 or GCN20. We also wanted to determine the effects of these mutations on activation of GCN2 and the GAAC response. From analysis of such mutations in conserved residues found in regions B and C, we have obtained strong evidence that GCN1-ribosome interaction is essential for GCN2 activation. Our results further demonstrate that specific residues in the eEF3-related domain of GCN1 function in signal transduction beyond their role in promoting ribosome binding by the GCN1-GCN2 complex.

### MATERIALS AND METHODS

**Yeast Strains and Plasmids—**The yeast strain used in this study was gen1Δ strain H2556 (MATa ura3–2, trp1–63 leu2–3, leu2–121 GAL2Δ (15)) constructed previously.² Plasmids used in this study are listed in Table I, and details of their construction are provided below. Vectors used were pRS316 and pRS425 (15).

For construction of the low copy plasmid pES176-4 containing gen1-MIA, two PCR amplifications were performed using p2367 as template, the primer pair ES110 and ES111, and the primer pair ES112 and ES109 (see Table II). In a subsequent PCR reaction, the two PCR amplifications were fused together using primers ES110 and ES109.

| Plasmid | Relevant gene under its own promotor | Relevant features | Vector | Source |
|---|---|---|---|---|
| p2367 | gcn1-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES176–4 | gcn1-M1A-myc² | URA3, CEN6/ARS4H | pRS316 | Ref. 8 |
| pES182–8 | gcn1-M7A-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES257–12–2 | gcn1-M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES263–34–1 | gcn1-M1A/M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| p1747 | GCN20 | LEU2, 2 μ | pRS425 | Ref. 8 |

² C. R. Vazquez de Aldana, and A. G. Hinnebusch, unpublished observations.

The XhoI-NruI-digested PCR fragment was ligated with plasmid p2367 digested with XhoI and NruI, yielding pES176-4. In this study, the sequences of all PCR-amplified inserts were verified.

**Insights into GCN1 mutations—**Table II provides a summary of the plasmids used in this study, along with the relevant gene or protein and its features. Relevant features are denoted as follows: (A) Apoptosis, (n) nMyc, (H) His, (E) eMyc, (M) Myc, and (H) His.

| Plasmid | Relevant gene under its own promotor | Relevant features | Vector | Source |
|---|---|---|---|---|
| pES176–4 | gcn1-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES182–8 | gcn1-M7A-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES257–12–2 | gcn1-M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES263–34–1 | gcn1-M1A/M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
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| pES182–8 | gcn1-M7A-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES257–12–2 | gcn1-M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| pES263–34–1 | gcn1-M1A/M7D-myc² | URA3, CEN6/ARS4H | pRS316 | This study |
| p1747 | GCN20 | LEU2, 2 μ | pRS425 | Ref. 8 |

² C. R. Vazquez de Aldana, and A. G. Hinnebusch, unpublished observations.

**Optimal GCN2 Activation Requires GCN1 Polyribosome Binding**
region for ribosome binding by \textit{S. cerevisiae} GCN1, we substitututed the 12 basic amino acids in this conserved motif with either uncharged Ala residues (the M7A mutation) or negatively charged Asp residues (M7D) and tested the full-length mutant GCN1 proteins for ribosome binding in yeast strains lacking chromosomal GCN1.

Cells expressing the mutant GCN1 proteins from alleles carried on low copy plasmids under the native GCN1 promoter were treated with formaldehyde to cross-link GCN1 to ribosomes in \textit{vivo} prior to preparing whole cell extracts (WCEs). The WCEs were resolved by velocity sedimentation through sucrose gradients, and the distributions of GCN1, GCN20, and the 60 S ribosomal subunit protein RPL39 across the fractions were determined by Western analysis. In the wild-type strain, GCN1 displayed a bimodal distribution. The majority of the protein and the largest polyribosomes were between fractions 13–15, and species sedimenting more slowly than 40 S subunits were contained in fractions 1–3 (Fig. 2A). GCN20 showed a similar distribution across the gradient, consistent with the idea that these two proteins bind to ribosomes as a complex (8). We showed previously that the fractions of GCN1 and GCN20 co-sedimenting with polyribosomes can be shifted to 80 S fractions by a light treatment of the WCE with micrococcal nuclease, which converts the polyribosomes to 80 S subunits, thus proving that GCN1 and GCN20 are associated with the polyribosomes (8).

To determine the effects of the M7A and M7D substitutions on polyribosome binding by GCN1 and GCN20, we quantified the Western signals in fractions 14 and 15 for each mutant, normalized them for the RPL39 signals in the same fractions, and expressed the normalized values as a fraction of the corresponding values measured for wild-type GCN1 (Fig. 2, B and C). (All GCN1 proteins showed a similar distribution in our sedimentation assays, with most of the polyribosomes and polyribosome-associated GCN1 and GCN20 in fraction 14 and 15.) From the results of two replicate experiments, we found that the M7A and M7D mutations reduced polyribosome binding by GCN1 to \textsim\,75 and \textsim\,40\% of wild type, respectively, and had similar effects on polyribosome binding by GCN20 (Fig. 2C). These results suggest that the basic residues in the conserved motif in area B of GCN1 are required for optimal ribosome binding by the GCN1-GCN20 complex \textit{in vivo}.

\textbf{Residues Conserved between GCN1 and eEF3 in Area C Cooperate with Basic Residues in Area B to Promote Ribosome binding by GCN1 \textit{in vivo}}—GCN1 has homology to the N terminus of eEF3, and the eEF3 N terminus (amino acids 98–388) interacts with 18 S rRNA \textit{in vitro} (19), suggesting that the eEF3-like region is involved in ribosome binding. In a past screen we attempted to identify amino acids in the eEF3-like region that are essential for GCN1 function. Sets of 5–10 amino acids conserved among the eEF3-like regions in GCN1 proteins

\begin{table}[h]
\centering
\caption{Primers used in this study}
\begin{tabular}{llll}
Primer name & Used to generate & Primer sequence$^a$ & Restriction site (position in primer/position in GCN1) \\
& gen1 allele & & \\
\hline
ES110 & gen1-M1A & gAT gCg ACC gCT CgA gCC & XhoI (11–16/4289–4294) \\
ES111 & gen1-M1A & gC gAg CgTAg CgC gAg CgT gCC gCT ggA & BamHI (24–29/4396–4401 in gen1-M1A and gen1-M1A/M7A only) \\
ES112 & gen1-M1A & CT ATT gCT gCT gCA gCT gCC gCT & \\
& & gCT gCT CA TAg TgC gAA CgA & \\
& & gCT ATT gCT TTC TTC gCT gA & \\
& & Anneals to GCN1 bases 4393–4351 & \\
& & Bases 1–19 anneal to ES111 bases 1–19 & \\
ES109 & gen1-M1A & gCCT gTCA AAg CgA CgT gAA gC & NruI (1–6/5223–5228) \\
ES900–17 & gen1-M7A & C AAA gAA CAT gCT AgC gTT ATC & NheI (11–16/2011–2016) \\
& gen1-M7D & & \\
ES900–19 & gen1-M7A & AC gCg ACC AAA TgC gTA Cg & MuI (17–19 part of site/3374–3379) \\
& gen1-M7D & & \\
ES900–9 & gen1-M7A & gTTC CCA CAT TAg AgT TTC gTA CTC & \\
& & gTg AgA ATT AgC ATT ggA AgA AgC & \\
& & ATT CAT gCT TTC TTC CgA gA & \\
& & Anneals to GCN1 bases 1149–2320 & \\
& & Bases 1–19 anneal to ES900–11 bases 1–19 & \\
ES900–10 & gen1-M7A & gAA ACT CTA TAg Tgg gAA CAA pCT & NotI (4:47/2341–2348 in gen1-M7A and gen1-M1A/M7A only) \\
& & pCT gAA ACT pAg CAA gAg gAg CgC & \\
& & gCT AAT gTg gCT gCT CTT gCA gCA & \\
& & gAA CAA gAg CTC gTT gAT & \\
& & Anneals to GCN1 bases 2299–2390 & \\
& & Bases 1–19 anneal to ES900–10 bases 1–19 & \\
ES900–11 & gen1-M7D & gTTC CCA CAT TAg AgT TTC gTA CTC & \\
& & gTA CTC AgA ATT ATT ATT gAA AgA AgC gTC & \\
& & ATT CAT gCT TTC TTC CgA gA & \\
& & Anneals to GCN1 bases 2320–2248 & \\
& & Bases 1–12 anneal to ES900–12 bases 1–22 & \\
ES900–12 & gen1-M7D & gAT gCA gCT gCT gAg CAA gAg & EcoRV (25–30/2323–2328 in gen1-M7D only) \\
& & gAT gCg gCC gCT & \\
& & gCg gCT gCT & \\
& & CT ATT gCT TTC & \\
& & CTT TCA gCA gA & \\
& & gAA CAA gAg CTC gTT gAT & \\
& & Anneals to GCN1 bases 2299–2391 & \\
& & Bases 1–22 anneal to ES900–11 bases 1–22 & \\
\hline
\end{tabular}
\end{table}

\begin{itemize}
\item $^a$Spacing indicates the reading frame.
\item $^b$Bases introducing the desired mutations are underlined.
\item $^c$Bases introducing a diagnostic restriction site are double underlined, and restriction site is in bold.
\end{itemize}
were substituted by Ala in full-length GCN1, and gcn1Δ strains harboring the corresponding mutant gcn1 alleles were scored for growth on the amino acid analog 3-amino-2,4-triazole (3AT). 3AT causes histidine starvation, and only strains able to activate GCN2 can overcome histidine starvation and grow on medium containing 3AT. In this screen (data not shown), we found that one of our gcn1 mutants rendered GCN1 non-functional in activating GCN2. This mutation contained six Ala substitutions in a block of conserved residues that were, in part, also conserved in eEF3 (the M1A allele, Fig. 1B). In this study, we asked whether these amino acids are involved in ribosome binding. We found that the M1 mutation led to reductions of 20–30% in polyribosome association of GCN1 (Fig. 2, A and B). Importantly, the M1A mutations exacerbated the polyribosome-binding defects of GCN1 and GCN2 produced by the M7 mutations described above, with the M1A/M7A double mutant showing substantially less GCN1-GCN20 binding to polyribosomes (~40% of wild type) than did the single mutants (70–80% of wild type) (Fig. 2C). These last findings suggest that the M1 and M7 residues function additively in promoting ribosome binding by GCN1.

Reduced Ribosome Binding of Mutant GCN1 Proteins Does Not Result from Defects in GCN1 Expression or GCN20 Interaction—We ruled out the possibility that the reduced ribosome binding of GCN1-GCN20 produced by the M1 and M7 mutations results from decreased expression of the mutant proteins by conducting Western blot analysis of WCEs prepared from the appropriate wild-type and gcn1 mutant strains (Fig. 3).

Previously, we presented evidence that ribosome binding by GCN1 is regulated by its interaction with GCN20 (5, 8); hence, it was important to determine whether the M1 and M7 mutations impair the GCN1-GCN20 interaction in vivo. This issue is particularly relevant for the M1 mutation because it alters residues in proximity to Gly-1444, shown previously to be required for interaction of GCN1 with GCN20. By conduct-
Additional evidence that the GCN1 mutations do not disrupt the GCN1-GCN20 complex is that they do not reduce the steady-state expression of GCN20 (Fig. 4A). We showed previously that substituting GCN1 residue Gly-1444 with Asp (the gcn1-D1444G allele) reduces GCN1-GCN20 interaction and greatly reduces the steady-state level of GCN20 (as does deletion of GCN1), presumably because GCN20 is more prone to degradation outside of the GCN1-GCN20 complex (8). Thus, the fact that the M1 and M7 substitutions do not reduce GCN20 levels further supports that GCN1-GCN20 interaction is unaffected by these GCN1 mutations.

We performed a final in vivo test to confirm that mutating the M1 residues does not impair GCN1-GCN20 interaction. The disruption of GCN1-GCN20 interaction by the gcn1-D1444G mutation impairs the GAAC response to histidine starvation (8). A defect in activation of GCN2 kinase function by GCN1-GCN20, with consequent failure to induce amino acid biosynthetic genes under its control, reduces cell growth on medium containing the inhibitor of histidine biosynthesis, 3AT. Interestingly, we found that the 3AT-sensitive phenotype of the gcn1-D1444G mutant, but not that of the gcn1Δ strain, is partially suppressed by GCN20 overexpression (Fig. 4C, cf. rows 1 and 4, upper panels). This suppression of gcn1-D1444G can be explained by proposing a partial restoration of complex formation between GCN20 and the gcn1-
D1444G product by mass action at elevated concentrations of GCN20. Importantly, the 3AT-sensitive phenotype of the gcn1-M1A strain was not suppressed by GCN20 overexpression, supporting our conclusion that substituting the M1 residues does not affect GCN20 binding activity.

Reduced GCN1 Ribosome Interaction in gcn1 Mutants Correlates with Reductions in GCN2 Activation and the GAAC Response—We have proposed that the GCN1-GCN20 complex must bind to the ribosome to facilitate activation of GCN2 by uncharged tRNAs bound to the ribosomal A-site of elongating ribosomes (5). If this model is correct, then the mutations in the M1 and M7 residues, which reduce GCN1 binding to ribosomes, should decrease phosphorylation of eIF2α by GCN2 and impair the GAAC response. To test this prediction, we compared the levels of phosphorylated eIF2α in WCEs of the mutant and wild-type strains by Western blot analysis using antibodies specific for eIF2α phosphorylated on Ser-51. We also measured the levels of total eIF2α using antibodies that recognize the protein regardless of its phosphorylation status (Fig. 5A). The amounts of eIF2α phosphorylated on Ser-51 were normalized for the corresponding amounts of total eIF2α and expressed relative to the normalized value measured for the GCN1 strain (Fig. 5B). In parallel, we compared the 3AT sensitivities of the same strains (Fig. 5C).

The M7A and M7D mutations in GCN1 produced reductions in eIF2α phosphorylation of ~20% (not statistically significant) and ~40%, respectively, in cells starved for histidine by 3AT treatment (Fig. 5B, cf. columns 2–4). The severity of these defects correlated well with the degrees of 3AT sensitivity exhibited by these two mutants (Fig. 5C, rows 1–3) and also with the relative defects in polyribosome binding by GCN1-GCN20 (Fig. 2C). These findings support the idea that polyribosome binding by GCN1 is critical for its ability to activate GCN2 and elicit the GAAC response. The M1A mutation had a stronger effect than either M7A or M7D on eIF2α phosphorylation and sensitivity to 3AT (Fig. 5, B and C), even though it closely resembled M7A with respect to the defect in polyribosome binding by GCN1-GCN20 (Fig. 2C). This last comparison suggests that the M1A mutation has a compound effect on GCN2 activation, impairing the ability of GCN1 to mediate GCN2 activation by uncharged tRNA in addition to reducing ribosome association by GCN1-GCN20. By contrast, the M7 mutations may impair only ribosome binding by GCN1-GCN20. Combining the M7A and M1A mutations produced a more severe reduction in eIF2α phosphorylation than did either single mutation, eliminating detectable amounts of eIF2α phosphorylated on Ser-51 (Fig. 5, A and B). This reduction is consonant with the additive reductions in GCN1 polyribosome association produced by combining these two mutations (Fig. 2D). The M1A/M7A double mutation did not confer greater sensitivity to 3AT than did the single M1A mutation, presumably because the latter is already indistinguishable from the gcn1Δ-null allele for this phenotype (Fig. 5C).
In this study, we characterized clustered substitutions in phylogenetically conserved residues located in two separate segments of GCN1 that lead to reductions in polyribosome association by the GCN1-GCN20 complex in vivo. Replacing the basic residues in the segment located in area B with Asp residues (mutation M7D) led to a greater reduction in polyribosome binding by GCN1-GCN20 than did alanine substitutions at the same residues (M7A), and it produced a correspondingly greater defect in both eIF2α phosphorylation by GCN2 and the GAAC response. In addition, combining Ala substitutions in the segment located in area C (M1A) with those in area B (M7A) led to additive effects in polyribosome binding by GCN1-GCN20, in eIF2α phosphorylation, and in the GAAC response. None of these mutations produced significant reductions in the steady-state levels of GCN1 or GCN20, nor did they impair GCN1-GCN20 or GCN1-GCN2 complex formation (Fig. 4A and data not shown). Together, these findings provide strong support for the proposition that binding of GCN1-GCN20 to polyribosomes is required for activation of GCN2 and the attendant induction of GCN4 translation in amino acid-starved cells. The fact that multiple, independent contacts seem to exist between GCN1 and the ribosome may reflect an interaction of GCN1 with distinct sites of the ribosome, e.g., on both 40 S and 60 S subunits, that contribute to stable association of the GCN1-GCN20 complex with the 80 S ribosome.

M1A and M7A mutations led to similar reductions in GCN1-GCN20 association with polyribosomes, but M1A produced a much greater defect in eIF2α phosphorylation than did M7A, which implies that the residues mutated by M1A in area C play an important role in signal transduction in addition to ribosome binding by GCN1-GCN20. Interestingly, these residues are located within a region of similarity between GCN1 and the N-terminal domain of eEF3. In view of existing evidence that eEF3 stimulates release of deacylated tRNA from the exit site and the binding of aminoacylated tRNA to the A-site (7), it is tempting to propose that interaction of the eEF3-related domain in GCN1 (region C) with the ribosome produces a conformational change in the A-site. This alteration could be instrumental in binding of uncharged tRNA to the A-site, or it could stimulate the transfer of uncharged tRNA from the A-site to the HisRS-like domain in GCN2 for kinase activation.

Previously, we found that significant binding of GCN1 and GCN20 to polyribosomes in WCEs was dependent on the addition of ATP to the extracts. In the current experiments, we measured GCN1-GCN20 binding to polyribosomes in WCEs lacking exogenous ATP that were prepared from cells treated with formaldehyde immediately before lysing the cells. Recently, we employed this cross-linking technique to preserve the labile association of translation initiation factors with ribosomal subunits during sedimentation through sucrose gradients, allowing us to assay translation initiation complex assembly in living yeast cells (16). Our finding of polyribosome association of GCN1-GCN20 in WCEs from cross-linked cells allows us to state unequivocally that these proteins are associated with translating ribosomes in vivo during steady-state growth in amino acid-replete cells. In addition, we can be certain that the M1A and M7A mutations reduce polyribosome association by GCN1-GCN20 in vivo by similar amounts, so the much stronger effect of M1A on GCN2 activation reflects a defect in signal transduction versus ribosome association of the regulatory complex. The use of formaldehyde cross-linking of living cells will be an indispensable tool for assigning specific residues in the eEF3-related domain to critical functions in ribosome binding versus GCN2 activation.

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