Molecular Cell, Vol. 6, 269–279, August, 2000, Copyright ©2000 by Cell Press

Uncharged tRNA Activates GCN2 by Displacing the Protein Kinase Moiety from a Bipartite tRNA-Binding Domain

Jinsheng Dong, Hongfang Qiu, Minerva Garcia-Barrio, James Anderson, and Alan G. Hinnebusch*
Laboratory of Eukaryotic Gene Regulation
National Institute of Child Health
and Human Development
Bethesda, Maryland 20892

Summary

Protein kinase GCN2 regulates translation in amino acid–starved cells by phosphorylating eIF2. GCN2 contains a regulatory domain related to histidyl-tRNA synthetase (HisRS) postulated to bind multiple deacylated tRNAs as a general sensor of starvation. In accordance with this model, GCN2 bound several deacylated tRNAs with similar affinities, and aminoacylation of $tRNA^{Phe}$ weakened its interaction with GCN2. Unexpectedly, the C-terminal ribosome binding segment of GCN2 (C-term) was required in addition to the HisRS domain for strong tRNA binding. A combined HisRS+C-term segment bound to the isolated protein kinase (PK) domain in vitro, and tRNA impeded this interaction. An activating mutation (*$GCN2^c$-E803V*) that weakens PK–C-term association greatly enhanced tRNA binding by GCN2. These results provide strong evidence that tRNA stimulates the GCN2 kinase moiety by preventing an inhibitory interaction with the bipartite tRNA binding domain.

Introduction

Protein synthesis in eukaryotic cells is inhibited in response to starvation, stress, or viral infection (Clemens, 1996) by phosphorylation of translation initiation factor 2 (eIF2) on serine 51 of its α subunit. Phosphorylation of eIF2 converts it from a substrate to an inhibitor of the guanine nucleotide exchange factor eIF2B, decreasing formation of the ternary complex (eIF2–GTP–Met-$tRNA_i^{Met}$) that transfers Met-$tRNA_i^{Met}$ to the ribosome. Four mammalian eIF2α kinases have been identified that are regulated by different stimuli: HRI (hemin deprivation), PKR (double-stranded RNA in virus-infected cells) (Clemens, 1996), PEK or PERK (unfolded proteins in the ER) (Shi et al., 1998; Harding et al., 1999), and mouse GCN2 (serum starvation) (Berlanga et al., 1998; Sood et al., 2000). There are also GCN2 homologs in *N. crassa* (Sattlegger et al., 1998), *D. melanogaster* (Santoyo et al., 1997; Olsen et al., 1998), and *S. cerevisiae*, where this enzyme was first discovered. Thus, GCN2 may be the most widespread and founding member of the eIF2α kinase subfamily (Dever, 1999).

The activation of PKR or HRI in mammalian cells inhibits general protein synthesis (Clemens, 1996). By contrast, activation of GCN2 in yeast cells stimulates translation of *GCN4* mRNA, encoding a transcriptional activator of amino acid biosynthetic enzymes. Four short open reading frames (uORFs) in the *GCN4* mRNA leader underlie a specialized reinitiation mechanism that elevates *GCN4* translation in response to small decreases in ternary complex levels insufficient to impair general translation. Thus, eIF2α phosphorylation in amino acid–starved yeast cells induces specifically a transcriptional activator that can rectify amino acid limitation. Dominant activating alleles of *GCN2* have been isolated that derepress *GCN4* under nonstarvation conditions, and the most severe of these $GCN2^c$ mutations inhibit general translation and cell growth due to high levels of eIF2α phosphorylation (Hinnebusch, 1996).

It is thought that GCN2 is activated by uncharged tRNAs that accumulate in amino acid–starved cells partly because mutations in aminoacyl tRNA synthetases (aaRSs) lead to GCN2-dependent derepression of *GCN4* without any starvation for amino acids (Hinnebusch, 1996). Additionally, GCN2 contains a region homologous to histidyl-tRNA synthetase (HisRS) located C-terminal to the kinase domain (Wek et al., 1989) (Figure 1A). Mutations in the GCN2 HisRS-like domain in conserved residues required for tRNA binding by authentic class II aaRSs (the m2 motif) inactivate *GCN2* function in vivo. These mutations also impair GCN2 kinase activity in cell extracts and destroy binding of tRNA to the isolated HisRS domain (Wek et al., 1995; Zhu et al., 1996). As *GCN4* translation is derepressed by starvation for any of several amino acids (Wek et al., 1995; Hinnebusch, 1996), the HisRS domain of GCN2 presumably cannot distinguish between different tRNA species and only discriminates against aminoacylated tRNAs. This prediction has not been tested, however, and it is possible that GCN2 binds charged and uncharged tRNAs alike but is activated only by the latter. The HisRS domain is conserved in all known GCN2 orthologs, suggesting that activation by uncharged tRNA is an evolutionarily conserved feature of these enzymes.

The extreme C-terminal segment of yeast GCN2 (C-term) is also essential for *GCN2* function in vivo (Wek et al., 1990). The C-term mediates ribosome binding in cell extracts (Ramirez et al., 1991; Zhu and Wek, 1998) and contains the principal dimerization determinants in GCN2 (Qiu et al., 1998) (Figure 1A). The isolated C-term can bind double-stranded RNA in vitro and interact with ribosomes in cell extracts, leading to the suggestion that it mediates ribosome binding by GCN2 through interactions with double-stranded segments of rRNA (Zhu and Wek, 1998). We showed recently that the extreme N terminus of GCN2 functions as the binding domain for the GCN1–GCN20 regulatory complex, which promotes the activation of GCN2 by uncharged tRNA in vivo (Garcia-Barrio et al., 2000).

In this report, we show that purified GCN2 interacts with multiple uncharged tRNAs with similar affinities but that it binds to aminoacylated $tRNA^{Phe}$ less tightly than to deacylated $tRNA^{Phe}$. These results provide direct evidence for two key postulates of our model. We found unexpectedly that the C-term is required in addition to the HisRS domain for strong tRNA binding by GCN2 in vitro. Remarkably, the isolated PK domain formed a stable complex with a fragment containing the combined HisRS+C-term regions, and this interaction was impeded by uncharged tRNA. Consistently, the HisRS+C-

*To whom correspondence should be addressed (e-mail: ahinnebusch@nih.gov).

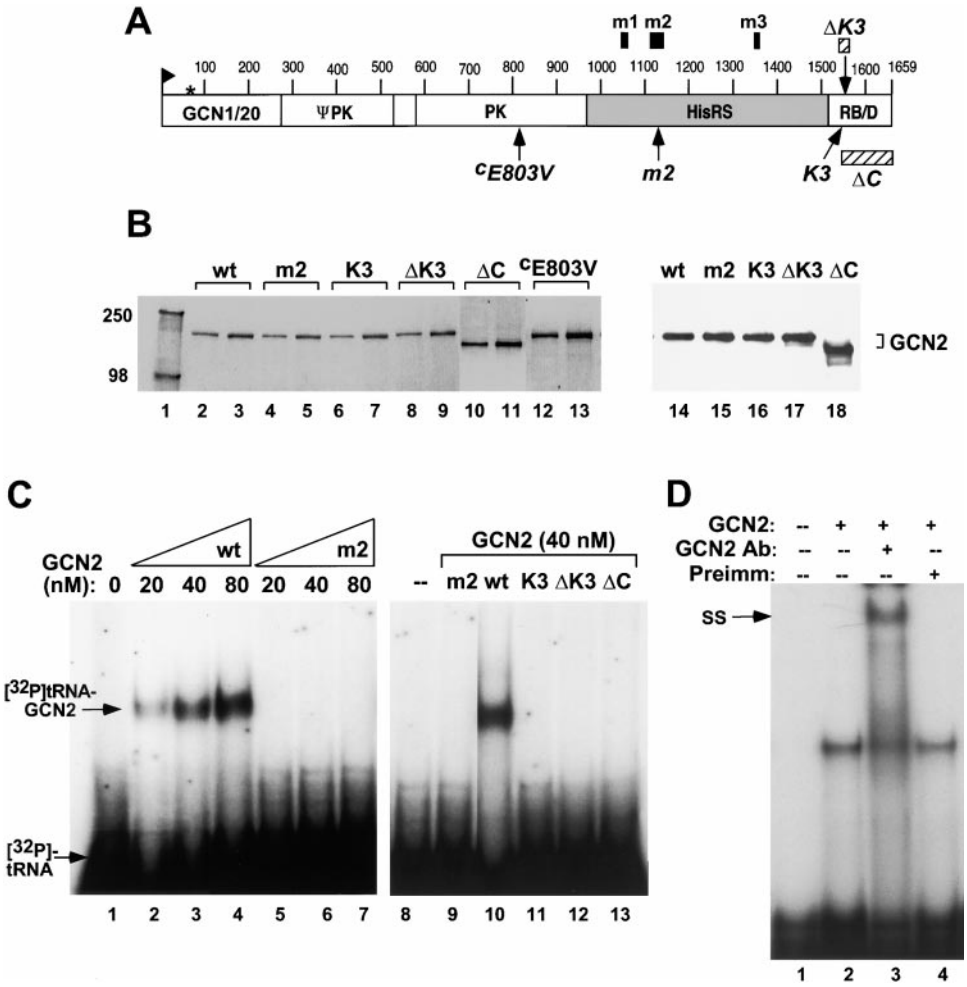

Figure 1. The C-Term Is Required in Addition to the m2 Motif of the HisRS-Related Region for tRNA Binding by GCN2

(A) The 1659 aa sequence of GCN2 is shown schematically with the following functional domains indicated: the binding domain for the GCN1–GCN20 complex, an uncharacterized pseudokinase domain (ΨPK), protein kinase domain (PK), HisRS-like region, and C-terminal ribosome binding and dimerization (RB/D) domains shown with shading or stippling. The flag at the N terminus designates the FLAG and polyhistidine tags incorporated into the protein for affinity purification. Above the schematic are listed the amino acid positions in GCN2 and locations of signature motifs (m1, m2, and m3) of class II aminoacyl-tRNA synthetases. The asterisk marks the methionine previously considered to be the first residue of GCN2 but now known to be residue 70. Arrows or hatched boxes above or below the schematic mark the locations of *gcn2* mutations *Y1119L,R1120L* (*m2*), *Δ1538–1557* (*ΔK3*), *K1552L, K1553I, K1556I* (*K3*), *Δ1536–1659* (*ΔC*), and *cE803V*.

(B) Characterization of affinity-purified GCN2 proteins. (Left) Samples of purified FLAG-tagged wild-type (wt) or mutant proteins designated as in (A) were resolved by 8% SDS–PAGE and stained with Coomassie Brilliant Blue (lanes 2–13). For each protein, 0.4 and 0.8 μg were loaded in successive lanes indicated with brackets. Lane 1 contains molecular weight markers. (Right) In lanes 14–18, 0.1 μg of the indicated proteins were resolved by 8% SDS–PAGE and subjected to immunoblot analysis using antibodies against GCN2 (HL2523).

(C) The purified wild-type (lanes 2–4) or m2 mutant proteins (lanes 5–7) described in (B) were incubated at the indicated concentrations with 20 nM of [^{32}P]-labeled total yeast tRNA in 20 μl of GMSA buffer (20 mM HEPES [pH 7.45], 150 mM NaCl, 7.5 mM $MgCl_2$, 10% glycerol, 0.2 U/μl Rnasin [Promega]). The GCN2–tRNA complexes were resolved by electrophoresis in a 6% polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer (3 hr, 120V), dried under vacuum, and visualized by autoradiography (the GMSA). In lanes 8–13, GMSA assays were performed using the indicated wild-type or mutant purified proteins at 40 nM.

(D) 2 μl of GCN2 antiserum (lane 3) or 2 μl of preimmune serum (lane 4) were added to a binding reaction containing 40 nM GCN2 and 20 nM [^{32}P]tRNA and subjected to GMSA, as in (C). The sample in lane 1 contained no GCN2. (SS), supershifted complex.

term segment bound tRNA much more tightly than did full-length GCN2. Moreover, an activating *GCN2c* mutation that weakened association between the PK and C-term segments greatly enhanced tRNA binding by the full-length protein. These findings indicate a straightforward mechanism for activation of GCN2 by uncharged tRNA, wherein tRNA binding eliminates an inhibitory physical interaction between the PK and HisRS+C-term domains.

Results

GCN2 Binds Different Uncharged tRNAs with Similar Affinities but Discriminates against Charged tRNA

To analyze the tRNA binding properties of GCN2 in vitro, we affinity-purified from yeast cells a tagged form of the protein (encoded by *GCN2-FL*) bearing the FLAG epitope and a polyhistidine stretch at the N terminus

Table 1. Plasmids

Plasmid	Description	Reference
pDH103	*GCN2-FL*, *FLAG-6* × His–tagged *GCN2* ORF under yeast *GAL* promoter in pEMBLyex4 (Cesareni and Murray, 1987); 2μ	This study
pDH104	*GCN2-FL-Y1119L R1120L (gcn2-FL-m2)* in pDH103 backbone	This study
pDH109	*GCN2-FL-K628R* in pDH103 backbone	This study
pDH110	*GCN2-FL-Δ1536–1659 (gcn2-FL-ΔC)* in pDH103 backbone	This study
pDH111	*GCN2-FL-K1552L K1553I K1556I (gcn2-FL-K3)* in pDH103 backbone	This study
pDH112	*GCN2-FL-Δ1538–1557 (gcn2-FL-ΔK3)* in pDH103 backbone	This study
pDH114	*GCN2-FL-E803V (GCN2-FL-^{c}E803V)* in pDH103 backbone	This study
p245	*HisRS+C-term (H+C)*, 6 × His–tagged *GCN2* codons 999–1659 under *E. coli lac* promoter	(Wek et al., 1995)
p274	*HisRS (H),* 6 × His–tagged *GCN2* codons 999–1536 under *E. coli lac* promoter	(Wek et al., 1995)
p297	*HisRS-Y1119L,R1120L (H-m2)* in p274 backbone	(Wek et al., 1995)
p245-m2	*HisRS+C-term-Y1119L,R1120L (H+C-m2)* in p245 backbone	This study
p245-K3	*HisRS+C-term-K1552L,K1553I,K1556I (H+C-K3)* in p245 backbone	This study
p245-ΔK3	*HisRS+C-term-Δ1538–1557 (H+C-ΔK3)* in p245 backbone	This study
pHQ298	*C-term* (*C*), 6 × His–tagged *GCN2* codons 1498–1659 under *E. coli lac* promoter	This study
pHQ551	*GST-PK*, GST fusion to *GCN2* codons 568–999 under *E. coli lac* promoter	(Qiu et al., 1998)
p331	*eIF2α–ΔC, SUI2* codons 1–199 under *E. coli lac* promoter	(Zhu et al., 1996)
pHQ400	*lexA-HA-GCN2*, LexA-, HA-tagged *GCN2* codons 27−1659 under yeast *ADH1* promoter, 2μ	(Qiu et al., 1998)
p630	Wild-type *GCN2*, 2μ	(Wek et al., 1990)
p2459	*gcn2-605* (Zhu and Wek, 1998) in p630 backbone	This study
pB91	*gcn2-Δ1538–1557* in p630 backbone	This study
p2461	*gcn2-Δ1536–1659* in p630 backbone	(Qiu et al., 1998)

(Table 1 and Figure 1B, [wt]). The two affinity tags did not interfere with *GCN2* regulatory function in vivo (data not shown). The purified GCN2 was tested for binding to total yeast tRNA by the gel mobility shift assay (GMSA) using gel-purified total yeast tRNA 5′ end–labeled with ^{32}P. As shown in Figure 1C, a discrete complex containing [^{32}P]tRNA was formed in a dose-dependent manner with GCN2 (wt). Addition of GCN2 antibodies to the binding reaction led to a novel complex of reduced electrophoretic mobility (supershift), verifying that the complex contained GCN2 (Figure 1D).

It was shown previously that the Y1119L and R1120L substitutions in the m2 motif of the HisRS-like domain impaired *GCN2* function in vivo (the *gcn2-m2* allele), and they abolished tRNA binding in a Northwestern assay by a recombinant polypeptide containing only the GCN2 HisRS-like domain (Wek et al., 1995). Accordingly, we purified the full-length GCN2 protein containing these mutations (the m2 mutant; Figures 1A and 1B) and examined it for complex formation with total tRNA in the GMSA. As shown in Figure 1C, the m2 protein failed to bind tRNA at 4-fold higher concentrations than were required to observe binding by wild-type GCN2. Mixing together equal amounts of the wild-type and m2 mutant proteins did not diminish complex formation by wild-type GCN2, ruling out the presence of an inhibitor in the mutant preparation (data not shown). We conclude that purified full-length GCN2 can form a specific complex with tRNA in a manner dependent on the m2 motif in the HisRS-like domain.

To test our prediction that GCN2 can bind different tRNAs with similar affinities, we compared individual unlabeled tRNAs for their ability to compete with total [^{32}P]tRNA for complex formation with GCN2. As shown in Figure 2C, addition of an ~100-fold excess of unlabeled total tRNA essentially eliminated complex formation between GCN2 and total [^{32}P]tRNA, whereas an equimolar amount of poly(A) RNA had no effect on the amount of GCN2–[^{32}P]tRNA complex formed. Gel-purified yeast tRNA^{Phe} and *E. coli* tRNA^{Tyr}, tRNA^{Lys}, and tRNA^{Val} each had competitor activity similar to that of total yeast tRNA (Figures 2A–2C). We also showed that each of these individual tRNAs, when end-labeled with ^{32}P, formed similar amounts of complex with GCN2 at equivalent concentrations of tRNA and protein (data not shown). We conclude that GCN2 has similar binding affinities for various individual uncharged tRNAs.

A second prediction of our model is that uncharged tRNA should bind more tightly than charged tRNA to GCN2. To test this prediction, purified yeast tRNA^{Phe} was aminoacylated with phenylalanine and compared with uncharged tRNA^{Phe} for competitor activity in the GMSA. Roughly 70% of the tRNA^{Phe} was aminoacylated under the conditions of our assays (data not shown). As shown in Figure 2D, the partially aminoacylated tRNA^{Phe} had substantially less competitor activity than did equivalent amounts of uncharged tRNA^{Phe}. To ensure that the tRNA^{Phe} was not damaged during aminoacylation, we deacylated the charged tRNA^{Phe} by alkaline hydrolysis and found that it recovered competitor activity comparable to the starting uncharged tRNA^{Phe} (Figure 2D). We conclude that aminoacylation of tRNA^{Phe} decreased its affinity for GCN2, in accordance with our model.

Lysine Residues in the C-Terminal Segment of GCN2 Are Required for Binding of Uncharged tRNA In Vitro

The C-term domain of GCN2 is required for kinase function in vivo (Wek et al., 1990; Zhu and Wek, 1998), for

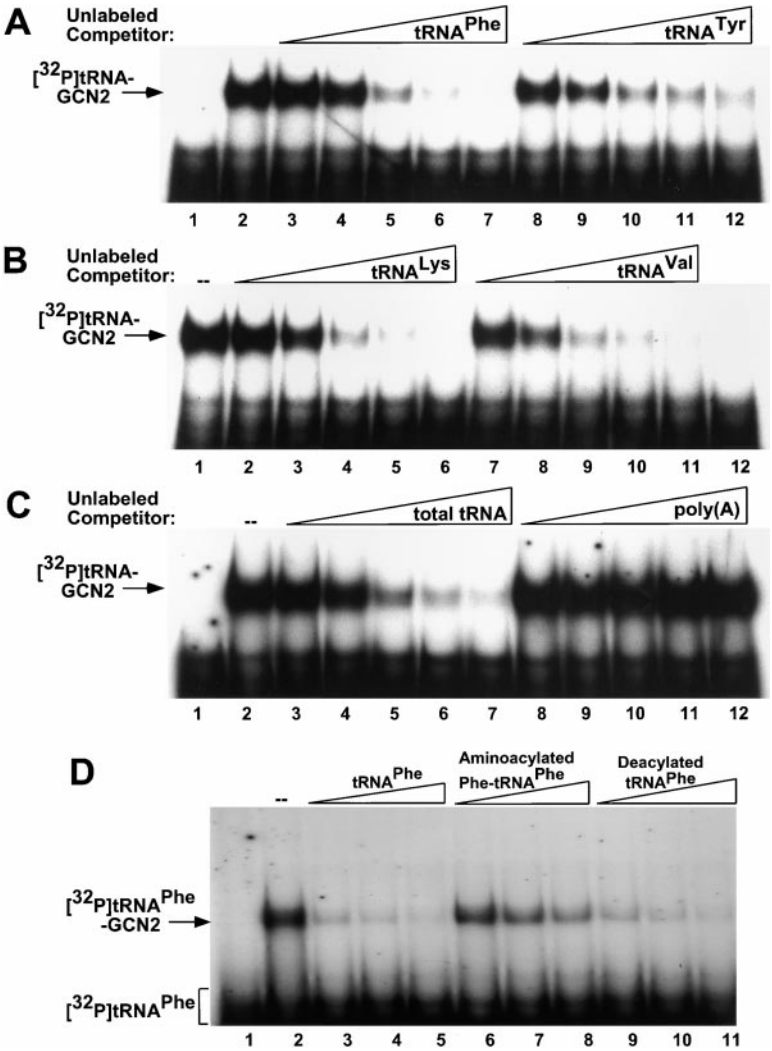

Figure 2. GCN2 Binds Different Uncharged tRNAs with Similar Affinities and Discriminates between Acylated and Deacylated $tRNA^{Phe}$

(A) [^{32}P]-labeled total yeast tRNA at 20 nM was incubated with purified GCN2 protein at 40 nM and either with no other tRNA (lane 2), with unlabeled yeast $tRNA^{Phe}$ at 40 nM, 160 nM, 640 nM, 1.28 μM, or 2.5 μM (lanes 3–7), or with unlabeled *E.coli* $tRNA^{Tyr}$ (lanes 8–12) at the same concentrations used for $tRNA^{Phe}$ in lanes 3–7, and subjected to the GMSA. The sample in lane 1 contained no GCN2.

(B and C) Reactions were carried out exactly as in (A) except using *E. coli* $tRNA^{Lys}$, *E. coli* $tRNA^{Val}$, total tRNA, or poly(A) RNA as unlabeled competitors, at the same concentrations given above for yeast $tRNA^{Phe}$. Lane 1 in (B) and lane 2 in (C) contained GCN2 but no competitor (−); lanes 12 and 1 in (B) and (C), respectively, contained no GCN2.

(D) [^{32}P]-labeled $tRNA^{Phe}$ at 40 nM and purified GCN2 at 120 nM were incubated with no other tRNA (lane 2, [–]), with 80 nM, 160 nM, or 240 nM of unlabeled $tRNA^{Phe}$ (lanes 3–5), with the same amounts of aminoacylated $tRNA^{Phe}$ (lanes 6–8), or with the same amounts of aminoacylated $tRNA^{Phe}$ deacylated prior to addition (lanes 9–11), and subjected to the GMSA. The sample in lane 1 contained no GCN2. Based on their relative abilities to compete with [^{32}P]-labeled $tRNA^{Phe}$ for binding to GCN2 and the percentage of $tRNA^{Phe}$ that was aminoacylated, we estimate that uncharged $tRNA^{Phe}$ binds GCN2 ~9-fold more tightly than does charged $tRNA^{Phe}$.

ribosome binding (Ramirez et al., 1991; Zhu and Wek, 1998), and for dimerization of full-length GCN2 molecules in cell extracts (Qiu et al., 1998). The location of several *GCN2^c* mutations in the C-term (Wek et al., 1990) suggested that this region also has a role in regulating kinase activity by uncharged tRNA. Accordingly, we asked whether a deletion that removes most of the C-term (Δ*1536–1659;* Figure 1A, [Δ*C*]) would alter the affinity of GCN2 for uncharged tRNA. We purified the product of *gcn2-FL-ΔC* (Figure 1B, [ΔC]) and found that it was completely defective for tRNA binding in the GMSA (Figure 1C, [ΔC]).

It was reported that a recombinant GCN2 C-term segment (residues 1536–1659) can bind to yeast ribosomes and has poly(I)–poly(C) binding activity dependent on lysine residues at positions 1552, 1553, and 1556. Substitutions of these Lys residues in full-length GCN2 with Leu, Ile, and Ile (the *gcn2–605* allele) impaired *GCN2* function in vivo and its association with ribosomes in cell extracts. Accordingly, it was proposed that these Lys residues mediate a critical interaction between the C-term and rRNA (Zhu and Wek, 1998). Having uncovered a requirement for the C-term in binding tRNA by GCN2, we asked whether Lys residues 1552, 1553, and 1556 also contribute to this activity. Mutant FLAG-tagged proteins containing the aforementioned substitutions in the Lys residues (K3 mutant) or a small deletion of amino acids 1538–1557 encompassing the Lys residues (ΔK mutant) were purified (Figures 1A and 1B) and tested for tRNA binding activity. Both the K3 and ΔK3 mutant proteins appeared to be completely defective for binding total [^{32}P]tRNA in the GMSA (Figure 1C). We conclude that Lys residues 1552, 1553, and 1556 in the C-term are required for tRNA binding by full-length GCN2, in addition to their role in ribosome binding.

To provide additional support for this last conclusion, we investigated the tRNA binding activities of recombinant fragments encompassing the GCN2 HisRS and C-term regions. Polypeptides bearing an N-terminal polyhistidine tag and either the HisRS and C-term regions combined (H+C), only the HisRS domain (H), or only the C-term (C) were expressed in *E. coli* and purified by nickel-affinity chromatography. The resulting preparations contained largely full-length forms of each recombinant protein (data not shown). The H+C fragment formed a stable complex with total [^{32}P]tRNA, whereas the m2 mutant derivative of this fragment (H+C-m2) was completely defective for tRNA binding (Figure 3A).

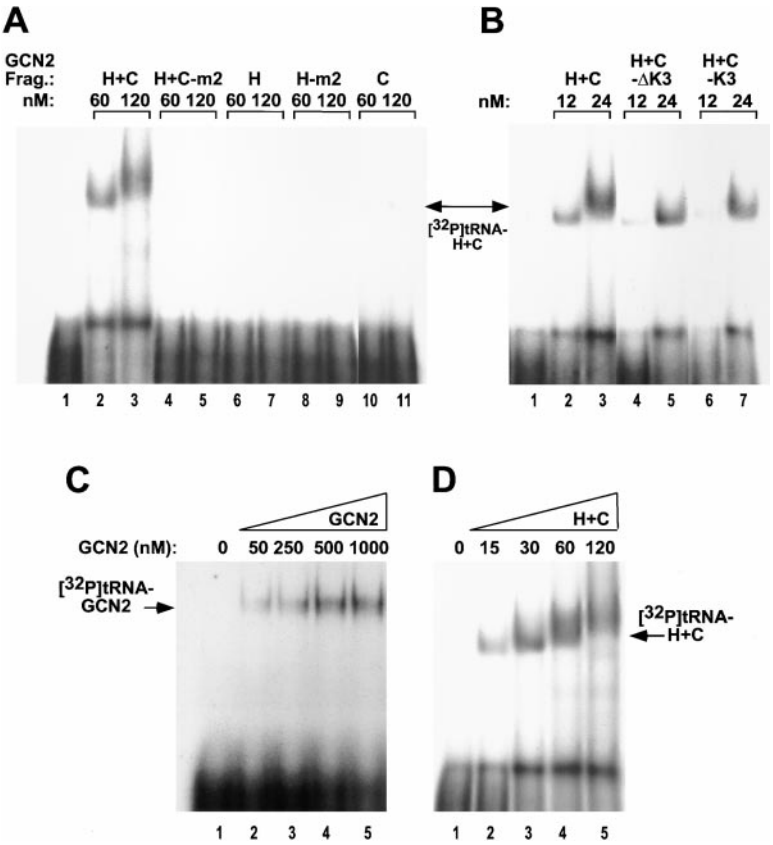

Figure 3. The HisRS+C-Term Fragment Expressed in *E. coli* Exhibits High-Affinity tRNA Binding Dependent on the C-Term

(A) [^{32}P]-labeled total yeast tRNA at 20 nM was incubated with 60 or 120 nM of the following polyhistidine-tagged GCN2 fragments purified from *E. coli* and subjected to the GMSA: the H+C polypeptide containing the HisRS and C-term domains (residues 999–1659) (lanes 2–3), a derivative of H+C bearing the *m2* mutations described in Figure 1 (HisRS+C-m2, lanes 4–5), the H polypeptide containing the HisRS-like region (residues 999–1536) (lanes 6–7), a derivative of H containing the *m2* mutation (H-m2, lanes 8–9), and the C-term polypeptide (residues 1498–1659) (C, lanes 10–11).

(B) [^{32}P]-labeled total yeast tRNA at 20 nM was incubated with 12 or 24 nM of H+C or its derivatives containing the Δ*K3* or *K3* mutations described in Figure 1 and subjected to the GMSA. Quantification of the tRNA–protein complexes by phosphorimaging analysis indicated that the wild-type H+C fragment bound roughly twice as much tRNA as did equivalent amounts of the H+C-ΔK3 or H+C-K3 proteins.

(C and D) [^{32}P]-labeled total yeast tRNA at 1 nM was incubated with full-length GCN2 protein at 50 to 1000 nM ([C], lanes 2–5) or with the H+C polypeptide at 15 to 120 nM ([D], lanes 2–5). The percentages of total [^{32}P]tRNA bound to the protein at each concentration were quantified by phosphorimaging, and the mean values from three replicate experiments had standard errors of <10%. The K_d value was equated with the protein concentration where 50% of the [^{32}P]tRNA was bound to protein.

Removal of the entire C-term from the H+C fragment also abolished tRNA binding, and the C-term alone had no binding activity (Figure 3A, fragment C). Introducing either the K3 or ΔK mutations into the H+C protein to remove Lys residues 1552, 1553, and 1556 reduced, but did not abolish, the tRNA binding activity of the H+C protein (Figure 3B; see legend). These results support the idea that these Lys residues in the C-term contribute to tRNA binding by GCN2. The residual binding activity of the H+C-K3 and H+C-ΔK3 mutant proteins compared to the inactivity of the H fragment (Figures 3A and 3B) could be explained by postulating an additional tRNA binding determinant in the C-term outside of the interval removed by the Δ*K3* mutation. Alternatively, the C-term may promote the tRNA binding activity of the HisRS domain by facilitating dimerization (Qiu et al., 1998) (see below).

Interestingly, we noted that the H+C fragment had a much greater affinity for tRNA than did full-length GCN2. Under conditions where the protein concentration was in large excess over the tRNA concentration, full-length GCN2 shifted the mobility of only ~30% of the total [^{32}P]tRNA at the highest protein concentrations we could achieve (1 μM) (Figure 3C), whereas the H+C fragment shifted ~50% of the probe at a protein concentration of only 60 nM (Figure 3D). Thus, the apparent dissociation constant (K_d) of the H+C–tRNA complex is ~60 nM, while the full-length GCN2–tRNA complex has an apparent K_d at least 20-fold larger. This difference in binding affinity may account for the fact that the Δ*K3* mutation abolished tRNA binding by full-length GCN2 (Figure 1C) but only weakened tRNA binding by the H+C fragment (Figure 3B).

The Lys Residues in the C-Term Are Required for Wild-Type Kinase Activity and Ribosome Association but Are Dispensable for Dimerization by GCN2

The substitution mutations of Lys residues 1552, 1553, and 1556 impaired *GCN2* function in vivo (Zhu and Wek, 1998), and the same was found for the Δ*K* mutation (Qiu et al., 1998). We assayed the kinase activities of the purified proteins bearing these mutations using [γ-^{32}P]ATP and a recombinant form of eIF2α (Zhu et al., 1996) as substrates. As shown in Figure 4A (right panel), the m2 mutant protein had substantially reduced autophosphorylation and eIF2α kinase activities compared to wild-type GCN2, but it retained more activity than the mutant protein containing Arg in place of the invariant lysine in kinase subdomain II (K628R) (Wek et al., 1989). Given that the *m2* mutation impairs tRNA binding, the reduced kinase activity of the m2 mutant suggests that tRNA had activated the wild-type protein in yeast cells prior to harvesting or during its purification. This interpretation is consistent with our observation that purified GCN2 could not be activated further by adding uncharged tRNA to the kinase assays at concentrations where most of the protein should be in a complex with

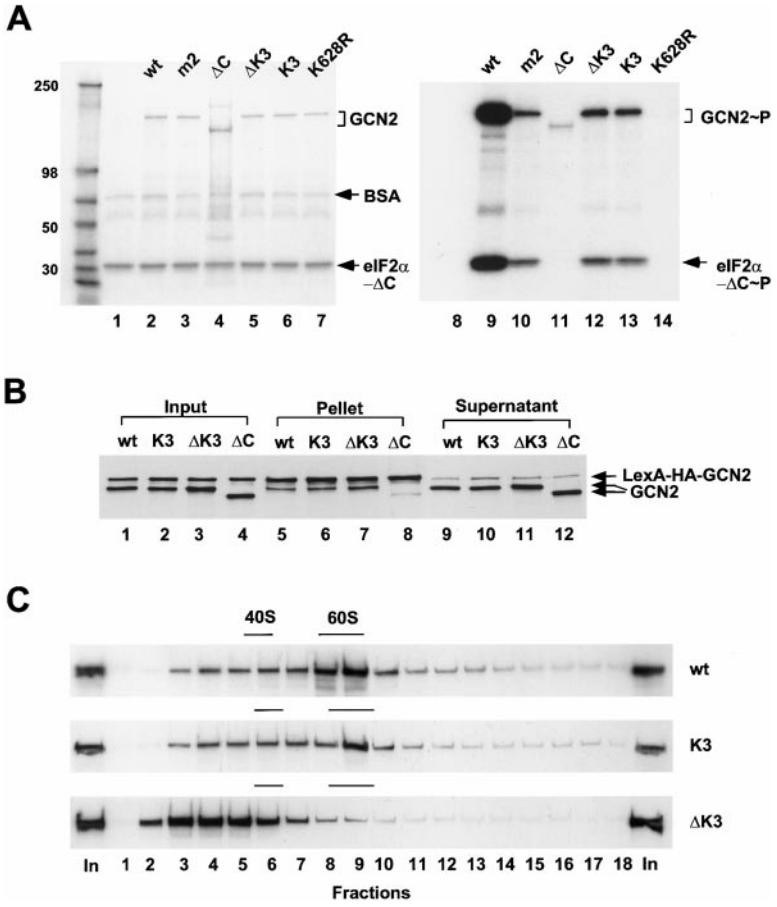

Figure 4. Substitution of Lysines in the C-Term Impairs Kinase Activity but Not Dimerization by Full-Length GCN2

(A) The purified GCN2 proteins (0.25 μg) designated as in Figure 1 were incubated with 3 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, Amersham), 1 μg of recombinant eIF2α−ΔC purified from *E. coli*, and 0.5 μg of bovine serum albumin in 20 μl of kinase assay buffer (20 mM Tris–HCl [pH 7.9], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM PMSF) for 15 min at 30°C. The samples were resolved by 8%–16% SDS–PAGE and stained with Coomassie blue (left), and the dried gel was subjected to autoradiography (right). Lanes 1 and 8 contained eIF2α−ΔC but no GCN2. The positions of phosphorylated GCN2 (GCN2∼P) and phosphorylated eIF2α−ΔC (eIF2α∼P) are indicated. The leftmost lane in the stained gel contained size standards.

(B) Coimmunoprecipitation assay of heterodimerization by GCN2 and lexA-GCN2 proteins in vivo. Extracts were prepared from transformants of *gcn2*Δ strain HQY132 bearing high-copy-number plasmid pHQ400 encoding LexA-HA-GCN2 and a high-copy plasmid bearing either wild-type *GCN2* (p630, wt), *gcn2–605* (p2459, K3), *gcn2-Δ1538–1557* (pB91, ΔK3), or *gcn2-Δ1536–1659* (p2461, ΔC), and aliquots with 50 μg of protein were incubated with Protein A agarose beads prebound with anti-HA antibody. The immune complexes were subjected to immunoblot analysis using anti-GCN2 antibodies. Lanes 1–4 contain 5 μg of the extracts used for immunoprecipitation (Input); lanes 5–8 contain samples of the pellet fractions corresponding to 25 μg of starting extract (Pellet); and lanes 9–12 contain samples of the supernatants corresponding to 5 μg of starting extract.

(C) Ribosome binding analysis. Cell extracts were prepared in a buffer lacking Mg^{2+} and cyclohexamide (to dissociate polysomes and 80S ribosomes into 40S and 60S subunits) from transformants of *gcn2*Δ yeast strain H1149 harboring high-copy plasmid p630 (wt), p2459 (K3), or p2453 (bearing *gcn2-K628R,Δ1538–1557*; ΔK3). Extracts were fractionated by velocity sedimentation in sucrose gradients, and the fractions were collected, monitored for A_{260}, and subjected to immunoblot analysis, using antibodies against the N terminus of GCN2. Fraction 1 is the top of the gradient. The first and last lanes (In) contain 1/50 of the starting extracts. Positions of free 40S and 60S subunits are indicated by bars at the top.

tRNA (data not shown). Similar findings were made previously for the *gcn2-m2* and *GCN2* products assayed in whole cell extracts (Zhu et al., 1996).

The K3 and ΔK3 mutant proteins also had reduced kinase activities relative to wild-type GCN2, (Figure 4A), consistent with their impaired abilities to interact with tRNA. The ΔC mutant had lower kinase activity than did the m2 protein (Figure 4A), suggesting that it lacks a function besides tRNA binding required for kinase activity. As the *ΔC* mutation impairs both ribosome binding (Ramirez et al., 1991) and dimerization (Qiu et al., 1998) of GCN2, one or both of these activities could be critically required for GCN2 activation by uncharged tRNA in extracts.

To explore further the basis for differences in kinase activity among the C-term mutants, we compared the ability of K3, ΔK3, and ΔC proteins to dimerize in vivo with a lexA-/HA epitope–tagged form of GCN2. In agreement with our previous findings (Qiu et al., 1998), the *ΔC* mutation substantially impaired dimer formation of GCN2 with lexA-HA-GCN2 (Figure 4B). In contrast, the K3 and ΔK3 proteins dimerized like wild-type GCN2 with lexA-HA-GCN2 (Figure 4B). We have also shown that a GCN2 C-terminal segment containing residues 1498–1659 can dimerize in the yeast two-hybrid assay (Qiu et al., 1998), and we verified that the K1552L, K1553I, K1556I triple substitution (*K3* mutation) had no effect on this two-hybrid interaction (data not shown). Thus, it is unlikely that removing Lys residues K1552, 1553, and 1556 from the C-term impairs GCN2 function in vivo by reducing dimerization.

It was shown previously that the triple Lys substitution in the C-term impaired binding of GCN2 to polysomes and 60S ribosomal subunits in cell extracts (Zhu and Wek, 1998). Binding of GCN2 to separated 40S and 60S subunits can also be assayed when extracts are prepared in the absence of Mg^{+2} to dissociate the polysomes (Ramirez et al., 1991). Under the latter conditions, we observed a relatively small defect in ribosome binding by the K3 mutant protein (Figure 4C). In contrast, the *ΔK3* deletion nearly eliminated binding of full-length GCN2 to isolated ribosomal subunits (Figure 4C). We suggest that there are more stringent requirements for the binding of GCN2 to polysomes versus separated

ribosomal subunits, and that Lys residues K1552, 1553, and 1556 comprise only a subset of the amino acids involved in ribosome binding. In this view, the Δ*K3* mutation would eliminate a larger number of residues required for the interaction and abolish ribosome binding by GCN2 as measured in both assays. Despite the fact that the *K3* mutation does not abolish ribosome binding, the deleterious effect of this mutation on kinase activity in vivo probably reflects a compound defect in ribosome association and tRNA binding by GCN2.

Uncharged tRNA Antagonizes Binding of the HisRS+C-Term Domain to the PK Domain of GCN2

The results in Figures 3C and 3D suggested that full-length GCN2 binds uncharged tRNA much less tightly than does the H+C fragment. A possible explanation for this phenomenon was prompted by our previous finding that the PK domain can physically interact with the isolated HisRS or C-term domains of GCN2 (Qiu et al., 1998). Thus, the PK domain and tRNA might compete for association with the HisRS-like and C-term regions. To test this idea, we asked whether the complex formed between the H+C polypeptide and total [^{32}P]tRNA could be dissociated by addition of a GST fusion to the PK domain. As shown in Figure 5A, addition of GST-PK at 16-fold molar excess over the H+C fragment greatly reduced the H+C–[^{32}P]tRNA complex detected by GMSA, whereas the same amounts of GST alone had no effect on H+C–[^{32}P]tRNA complex formation. As expected, GST-PK could not form a stable complex with [^{32}P]tRNA (Figure 5A).

To provide additional evidence that tRNA and PK compete for interaction with the H+C fragment, we asked whether total uncharged tRNA could impede GST-PK–H+C complex formation as assayed by precipitation on glutathione-Sepharose beads (GST pull-down). We found that GST-PK, but not GST alone, formed a stable complex with the H+C fragment in the absence of tRNA (Figure 5B). Addition of total tRNA at a 100-fold molar excess over the H+C fragment dissociated the GST-PK–H+C complex. The H+C-m2 fragment also formed a stable complex with GST-PK, but in this case, addition of excess tRNA led to only a slight reduction in GST-PK–H+C-m2 complex formation (Figure 5B). This last finding supports the notion that motif 2–dependent binding of tRNA to the H+C fragment is responsible for dissociation of the GST-PK–H+C complex. Similarly, we found that the H+C-K3 protein could form a stable complex with GST-PK that was relatively resistant to challenge with tRNA, showing only an ~50% reduction in the amount of complex formed at the highest tRNA concentration tested (data not shown). Finally, we observed that purified tRNA^{Phe} was comparable to total tRNA in the ability to dissociate the GST-PK–H+C protein complex, whereas aminoacylated Phe-tRNA^{Phe} had little or no dissociating activity in this assay (Figure 5B). The results in Figures 5A and 5B provide strong evidence that binding of uncharged tRNA to the H+C fragment weakens interaction of the latter with the PK domain. This finding leads us to suggest that tRNA binding will release the PK domain from inhibitory interactions with the HisRS or C-term segments in the context of full-length GCN2.

In vivo evidence supporting this hypothesis emerged from our analysis of the *GCN2^{c}-E803V* allele, bearing a point mutation in the PK domain (Figure 1A). This mutation elicits high-level eIF2α phosphorylation by GCN2 under nonstarvation conditions, where uncharged tRNA is scarce (Ramirez et al., 1992). Interestingly, the *^{c}E803V* substitution impaired physical interaction between the PK and C-term domains in yeast two-hybrid assays and in vitro binding assays (H. Q. and A. G. H., unpublished data). This last finding suggests that the *E803V* mutation activates GCN2 by decreasing association between the PK and C-term domains. Our model predicts that weaker interaction between the C-term and PK domains should enhance tRNA binding by decreasing competition between the tRNA and PK domains for binding to the HisRS+C-term domain. In agreement with this prediction, the purified GCN2-^{c}E803V protein (Figure 1B) showed a much higher affinity for tRNA compared to wild-type GCN2 (Figure 5D). Using low concentrations of [^{32}P]tRNA in the GMSA, we estimated the K_d for GCN2-^{c}E803V at ~100 nM, more than 10-fold lower than that of wild-type GCN2 and only slightly higher than that of the recombinant H+C fragment. The enhanced affinity of GCN2^{c}-E803V for uncharged tRNA provides a satisfying explanation for its ability to be activated in nonstarved yeast cells.

Discussion

GCN2 Preferentially Binds Uncharged tRNAs

GCN2 becomes activated in response to starvation for any single amino acid by binding the uncharged cognate tRNAs that accumulate in starved cells. Direct involvement of the HisRS-like domain in binding uncharged tRNA was demonstrated previously, and mutational analysis of the m2 motif provided strong evidence that tRNA binding to this domain is required for GCN2 activation in starved cells (Wek et al., 1995; Zhu et al., 1996). Using highly purified preparations of full-length GCN2, we obtained biochemical evidence that the HisRS-like domain can bind different individual tRNAs with similar affinities, and that aminoacylation of tRNA reduces its affinity for GCN2. Thus, the ability of deacylated tRNAs to activate GCN2 may reside simply in their higher affinities, relative to the corresponding acylated forms, for the tRNA binding domain in GCN2.

The affinity of wild-type GCN2 for uncharged tRNA is fairly similar to that of authentic *E. coli* HisRS (Bovee et al., 1999). Whereas aaRSs discriminate strongly against certain noncognate tRNAs at the binding step, in other cases noncognate and cognate tRNAs bind with similar affinities and discrimination occurs at the step of catalysis or product release (Schimmel and Soll, 1979). Interestingly, *E. coli* HisRS binds tRNA^{His} only slightly stronger than it does tRNA^{Phe} (Bovee et al., 1999); thus, GCN2 may resemble authentic HisRS in binding different uncharged tRNAs with similar affinities. To account for the reduced affinity of GCN2 for charged tRNA, we imagine that the segment of the HisRS-like region corresponding to the active site cavity does not contain a pocket that can accommodate an amino acid attached to the acceptor stem of the tRNA. In an authentic class II enzyme, the binding pocket for the amino acid contains residues that provide Van der Waals contacts, hydrogen bonds, or electrostatic interactions with the backbone and side chain of the amino acid (Ruff et al., 1991; Arnez et al., 1995). Some of these residues are replaced with nonconserved amino acids in the HisRS-like domain of GCN2

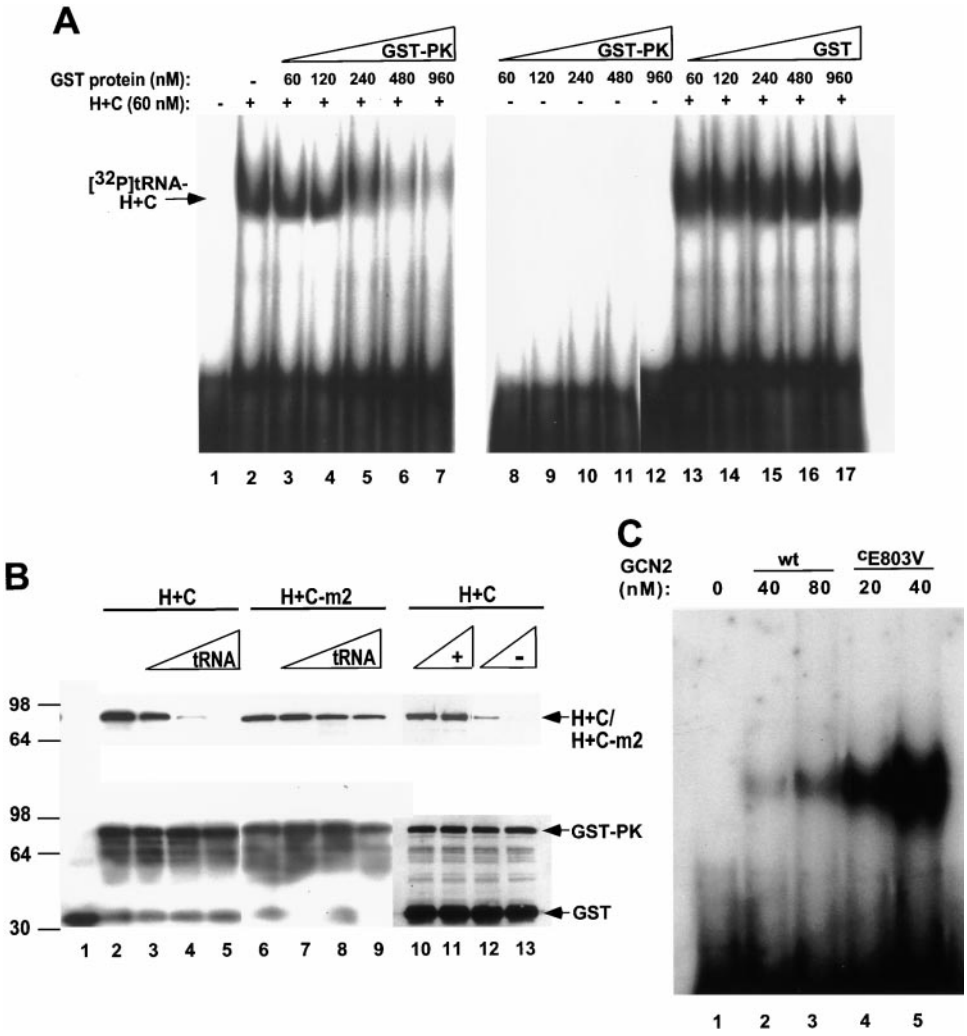

Figure 5. The PK Domain Competes with tRNA for Association with the HisRS+C-Term Segment of GCN2

(A) [^{32}P]-labeled tRNA at 20 nM was incubated with the H+C polypeptide at 60 nM and either with no other protein (lane 2), with the indicated amounts of GST-PK fusion bearing GCN2 residues (568–999) (lane 3–7), or with GST alone (lanes 13–17) and subjected to the GMSA. In lanes 8–12, GST-PK was present but H+C was omitted.

(B) GST-PK at 1.1 μM (lanes 2–9) or GST alone at 2 μM (lane 1) immobilized on glutathione-Sepharose beads was incubated with the H+C polypeptide at 1.2 μM (lanes 2–5) in the absence (lane 2) or presence of 30 μM, 60 μM, or 120 μM total yeast tRNA (lanes 3–5, respectively) at 4°C for 1 hr. Lanes 6–9 were identical to lanes 2–5 except that 1.2 μM H+C-m2 polypeptide replaced the H+C polypeptide. Lanes 10–11 and 12–13 were identical to lanes 4–5 except that 60 μM or 120 μM of aminoacylated tRNA^{Phe} (lanes 10–11; [+]) or the same concentrations of nonacylated tRNA^{Phe} (lanes 12–13; [–]) replaced total tRNA. After extensive washing, the bound proteins were eluted by boiling in sample buffer and subjected to immunoblot analysis with antibodies against the polyhistidine tag (Santa Cruz) (top), then stripped and reprobed with anti-GST antibodies (Santa Cruz) (bottom). Numbers on the left indicate the mobilities of size standards with the indicated molecular weights in kDa.

(C) The indicated purified GCN2 proteins were subjected to GMSA as in Figure 1C.

(Ramirez et al., 1992; Arnez et al., 1995), perhaps accounting for its reduced interaction with charged versus uncharged tRNA^{Phe}.

The C-Terminal Ribosome Binding/Dimerization Domain of GCN2 Is Also Required for tRNA Binding

We found unexpectedly that lysine residues 1552, 1553, and 1556 in the C-term are required for tRNA binding to the HisRS-like domain of GCN2. Substitutions of these residues abolished tRNA binding by full-length GCN2 and reduced the binding activity of the recombinant HisRS+C-term (H+C) fragment. Mutations in motif 2 had a more severe effect than did removal of the C-term Lys residues, abolishing tRNA binding by both the H+C polypeptide and full-length GCN2. However, a recombinant polypeptide containing the HisRS-like domain alone (the H fragment) could not form a stable complex with tRNA, demonstrating an absolute requirement for the C-term in tRNA binding.

The greater tRNA binding activity of the H+C fragment lacking only the three Lys residues (H+C-K3) versus the H fragment alone could reflect the presence of additional tRNA binding determinants in the C-term. Alternatively, the C-term may enhance tRNA binding by promoting dimerization of the HisRS region, as it contains a dimerization interface and also binds to the HisRS region (Qiu et al., 1998). This last explanation might account for the

fact that the H polypeptide was shown previously to bind tRNA in the Northwestern assay (Wek et al., 1995). Perhaps when the H fragment is concentrated by gel electrophoresis and tethered to a filter, dimerization of the HisRS-like segment can occur independently of the C-term and permit a reduced level of tRNA binding similar to that observed for the H+C-K3 and H+C-ΔK3 fragments lacking the C-term Lys residues.

Mutating Lys residues 1552, 1553, and 1556 did not impair dimerization by the isolated C-term or by full-length GCN2. Thus, the reductions in tRNA binding and GCN2 kinase activity associated with these particular C-term mutations probably did not arise from reduced dimerization. Wek and colleagues showed that the C-term lysines are required for binding of full-length GCN2 to polysomes (Zhu and Wek, 1998). We found that substitutions in the C-term lysines impaired but did not abolish ribosome binding, leading us to suggest that the strong requirement for these residues for *GCN2* function in vivo additionally reflects their contribution to tRNA binding. Accordingly, we propose that the HisRS and adjacent C-term regions comprise a bipartite tRNA binding domain, and that the C-term has an additional function in ribosome binding. In class II aminoacyl-tRNA synthetases, the acceptor stem of the tRNA interacts with motif 2 (Ruff et al., 1991). It is tempting to propose that the C-term of GCN2 provides additional contacts with the anticodon stem in the manner proposed for authentic HisRS (Arnez et al., 1995; Wek et al., 1995) (Figure 6). In this view, eliminating contacts with the acceptor stem by mutating motif 2 residues or with the anticodon stem by mutating the C-term lysines each would destabilize the GCN2–tRNA complex.

tRNA and the Kinase Domain Compete for Association with the HisRS+C-Term Domain

We previously detected physical interactions between the kinase domain of GCN2 and both the HisRS-like and C-term segments by two-hybrid and in vitro binding assays (Qiu et al., 1998). Given that these three segments harbor all of the known *GCN2*^{c} regulatory mutations (Wek et al., 1990; Ramirez et al., 1992), we speculated that binding of the HisRS or C-term domains to the PK moiety could underlie the negative regulation of kinase activity in nonstarved cells. These negative interactions would be overcome through binding of uncharged tRNA to the HisRS-like domain in starved cells (Qiu et al., 1998). Here, we obtained direct evidence supporting this hypothesis by showing that tRNA can dissociate the complex formed between a GST-PK fusion and the H+C polypeptide. Thus, tRNA and the PK domain may compete for certain binding sites in the HisRS+C-term domain. This possibility is consistent with our finding that full-length GCN2 had a much lower affinity for tRNA than did the H+C polypeptide, as the presence of the PK domain in native GCN2 would be expected to antagonize its interaction with tRNA. Based on these findings, we propose that the combined HisRS+C-term region is bound to the kinase domain in nonstarved cells, where uncharged tRNA is scarce, and that these protein–protein interactions contribute substantially to the inhibition of kinase activity under these conditions. The higher concentration of uncharged tRNA in starved cells would favor tRNA binding to the HisRS+C-term, eliminating inhibitory contacts of the latter with the kinase domain and thereby activating GCN2 (Figure 6).

Strong in vivo evidence supporting this model was provided by our finding that the GCN2^c-E803V protein had a much greater affinity for tRNA than did wild-type GCN2. The ^{c}*E803V* mutation in the PK domain leads to activation of GCN2 under nonstarvation conditions (Ramirez et al., 1992), and this substitution impairs physical association of the PK and C-term domains (H. Q. and A. G. H., unpublished data). If the PK domain and tRNA compete for binding to the HisRS+C-term, then by weakening the association between PK and C-term the ^{c}*E803V* mutation should enhance tRNA binding by GCN2, as we observed. The increased affinity of purified GCN2^c-E803V for uncharged tRNA can account for its activation in nonstarved yeast cells when uncharged tRNA is scarce. Our observations that the ^{c}*E803V* mutation weakens PK–C-term interactions, enhances tRNA binding in vitro, and leads to constitutive activation of GCN2 in vivo provide a compelling combination of genetic and biochemical evidence supporting our model for GCN2 activation.

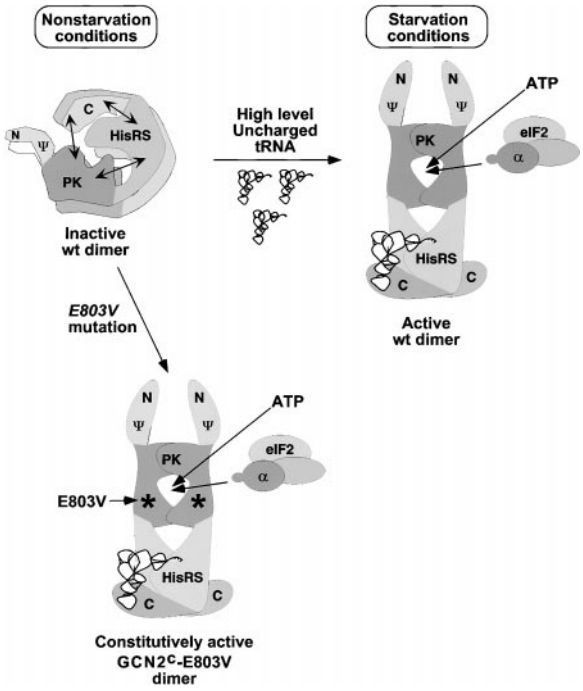

Figure 6. Model for Activation of GCN2: Dissociation of Autoinhibitory tRNA Binding Domain from the Kinase Domain Triggered by Binding of Uncharged tRNA

Domains of GCN2 are labeled as follows: GCN1–GCN20 binding domain at the N terminus (N), pseudokinase domain (Ψ), protein kinase domain (PK), HisRS-like region (HisRS), and C-term (C). Both the inactive (left) and activated forms of wild-type GCN2 are depicted as dimers because the tRNA binding (HisRS) domain is dispensable for dimerization; thus, it is unlikely that dimerization is triggered by the uncharged tRNA that builds up in starved cells. Dimerization is driven primarily by the C-term segment, but also occurs in the PK domain. The C-term and HisRS segments are responsible for autoinhibitory interactions with the kinase domain (depicted by double-headed arrows). For wild-type (wt) GCN2, binding of uncharged tRNA to the HisRS region and C-term segments triggers a conformational change that disrupts the inhibitory PK–C-term and PK–HisRS interactions (right) to permit phosphorylation of the substrate eIF2. The GCN2^c-E803V protein binds tRNA and is activated under nonstarvation conditions where uncharged tRNA is scarce (left) because the E803V mutation weakens the PK–C-term interaction, reducing the ability of the PK domain to compete with tRNA for binding to the HisRS+C-term domains.

We have argued previously that uncharged tRNAs bound to the decoding (A) site of the ribosome and base-paired with their cognate codons in mRNA activate GCN2 (Ramirez et al., 1991). A similar mechanism operates in the stringent response of *E. coli* where the ppGpp synthetase relA is activated by uncharged tRNAs in the A site (Cashel and Rudd, 1987). It is intriguing that the C-term of GCN2 promotes both ribosome association (presumably via rRNA binding) and tRNA binding, but it is presently unclear whether these interactions are mutually exclusive. If the contacts between GCN2 and rRNA must be severed to accommodate binding of tRNA, this would lead to dissociation of the activated GCN2–tRNA complex from the ribosome. While ribosome binding by GCN2 would be required for interaction with uncharged tRNA in the A site, its subsequent release from the ribosome may enhance the ability to bind and phosphorylate eIF2.

Experimental Procedures

Plasmids and Strains

The plasmids employed are described in Table 1. Details of their construction will be provided on request. Yeast strains GP3299 (Pavitt et al., 1997), HQY132 (Qiu et al., 1998), and H1149 (Wek et al., 1990) were described previously.

Protein Purification

Transformants of strain GP3299 (*a ura3–52 leu2–3 leu2–112 GCD2-K627T gcn2*Δ) bearing plasmids pDH103, pDH104, pDH109, pDH110, pDH111, pDH112, or pDH114 (Table 1) were grown to saturation in SC-Ura medium, diluted to OD_{600} = 0.4 in minimal medium containing 10% galactose as carbon source and minimal supplements, and grown to OD_{600} ~0.9, whereupon 10 mM 3-AT was added. At OD_{600} of ~2.5, cells were harvested (~25 g) and washed with cold distilled water containing EDTA-free protease inhibitor cocktail (PIC) (Boehringer Mannheim) and 0.5 mM PMSF, resuspended in ice-cold binding buffer (BB) (100 mM sodium phosphate [pH 7.4], 500 mM NaCl, 0.1% Triton X-100, EDTA-free PIC, 1 μg/ml leupeptin, and 1 mM PMSF), and disrupted with a French Press. Lysates were clarified by centrifugation at 39,000 × g for 2 hr at 4°C and mixed with 1 ml of M2-FLAG affinity resin (Sigma) for ~4 hr at 4°C. The resin was washed three times with 10 vol of BB and eluted with 3 vol of 2 mM FLAG peptide in BB, followed by two more volumes of the same. The eluates were pooled and concentrated with an Amicon Centricon filter (exclusion limit of M_r 10,000). For nickel affinity purification, 100 μl of Ni-NTA Agarose (Qiagen) was washed twice with 500 μl of BB containing 5 mM imidazole for 30 min and mixed with FLAG-affinity-purified GCN2 at 4°C for 1 hr, washed three times with 500 μl of BB containing 30 mM imidazole, and eluted twice with 200 μl of BB containing 250 mM imidazole. The eluates were concentrated as above, dialyzed against 100 mM sodium phosphate (pH 7.4), 100 mM NaCl, and stored at −80° C in 100 mM sodium phosphate (pH 7.4), 50% (v/v) glycerol. The polyhistidine-tagged GCN2 fragments (Wek et al., 1995), GST and GST-PK fusion proteins (Qiu et al., 1998), and the eIF2α−ΔC (Zhu et al., 1996) protein were purified from *E. coli* as previously described.

Gel Mobility Shift Assays of GCN2–tRNA Complexes

Total yeast tRNA was purchased from Roche, purified individual tRNAs were purchased from Sigma, and poly(A) RNA was obtained from Pharmacia. All of the tRNAs were gel purified as described previously (Seong and RajBhandary, 1987). The tRNAs were 5′ end–labeled by dephosphorylating with calf intestine alkaline phosphatase (New England BioLabs) and then phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. The GMSA was conducted essentially as described previously (Simos et al., 1996). The [^{32}P]tRNA–GCN2 complexes were quantified with a phosphorimager (Molecular Dynamics) using the Image Quant software provided by the vendor. For the supershift assay, GCN2 antiserum (HL2523) (Garcia-Barrio et al., 2000) or preimmune antiserum was purified with Avid-Almatrix (Unisyn Technologies) according to the vendor's instructions, and 2 μl were added to the binding reactions prior to electrophoresis.

Aminoacylation of Yeast tRNA^{Phe}

Aminoacylation was carried out at 37°C for 30 min in a reaction mixture containing 100 mM Tris–HCl (pH 7.4), 12 mM ATP, 40 mM $MgCl_2$, 2 μM amino acid mixture minus Phe (Sigma), 0.2 μM L-[2,3,4,5,6-^{3}H]Phe (122 Ci/mmol; Amersham), 1.8 μM Phe, 0.25 μM purified tRNA^{Phe}, and a 40 μl/ml crude preparation of rabbit aminoacyl-tRNA synthetases (Hatfield et al., 1979). The Phe-tRNA^{Phe} was extracted with an equal volume of extraction buffer (10 mM sodium acetate [pH 4.5], 10 mM $MgCl_2$, 1 mM EDTA, and 450 mM NaCl) and water-saturated phenol, ethanol precipitated, and resuspended in 1 ml of buffer F (100 mM sodium acetate [pH 5.0], 1 mM dithiothreitol). The tRNAs were desalted on a G-25 column equilibrated with 0.1 mM potassium cacodylate (pH 5.5) at 4°C, lyophilized, resuspended in buffer F, and stored at −80°C. Scintillation counting of the final preparation indicated that a minimum of 70% of the tRNA^{Phe} was aminoacylated. Deacylation of the Phe-tRNA^{Phe} was carried out by adding Tris–HCl (pH 9.0) to 1.8 M and incubating at 30°C for 30 min, during which >95% of the charged tRNA was deacylated, as monitored by filter binding assays and scintillation counting. The deacylated tRNA was ethanol precipitated and resuspended in H_2O at the same concentration as the starting sample of acylated tRNA^{Phe}.

Analysis of Dimerization and Ribosome Binding by GCN2 In Vivo

Assays for the presence of heterodimers containing GCN2 and lexA-GCN2 proteins by coimmunoprecipitation analysis (Qiu et al., 1998) and of polysome binding by GCN2 (Ramirez et al., 1991) in yeast cell extracts were conducted as previously described.

Acknowledgments

We are indebted to Ron Wek and colleagues for providing numerous constructs, assistance, and comments on the manuscript. We also thank Tom Dever for critical reading of the manuscript and colleagues in the LEGR for suggestions and discussions. J. D. was supported as a Research Associate by the National Research Council.

Received March 3, 2000; revised June 12, 2000.

References

Arnez, J.G., Harris, D.C., Mitschler, A., Rees, B., Francklyn, C.S., and Moras, D. (1995). Crystal structure of histidyl-tRNA synthetase from *Escherichia coli* complexed with histidyl-adenylate. EMBO J. *14*, 4143–4155.

Berlanga, J.J., Herrero, S., and de Haro, C. (1998). Characterization of the hemin-sensitive eukaryotic initiation factor 2α kinase from mouse nonerythroid cells. J. Biol. Chem. *273*, 32340–32346.

Bovee, M.L., Yan, W., Sproat, B.S., and Francklyn, C.S. (1999). tRNA discrimination at the binding step by a class II aminoacyl-tRNA synthetase. Biochemistry *38*, 13725–13735.

Cashel, M., and Rudd, K.E. (1987). The stringent response. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, F.C. Neidhardt, J.L. Ingraham, B. Magasanik, K.B. Low, M. Schaechter and H.E. Umbarger, eds. (Washington, DC: American Society for Microbiology), pp. 1410–1438.

Cesareni, G., and Murray, J.A.H. (1987). Plasmid vectors carrying the replication origin of filamentous single-stranded phages. In Genet. Eng.: Principals and Methods, J.K. Setlow and A. Hollaender, eds. (New York: Plenum Press), pp. 135–154.

Clemens, M.J. (1996). Protein kinases that phosphorylate eIF2 and eIF2B, and their role in eukaryotic cell translational control. In Translational Control, J.W.B. Hershey, M.B. Mathews and N. Sonenberg, eds. (Plainview, NY: Cold Spring Harbor Laboratory Press), pp. 139–172.

Dever, T.E. (1999). Translation initiation: adept at adapting. Trends Biochem. Sci. *24*, 398–403.

Garcia-Barrio, M., Dong, J., Ufano, S., and Hinnebusch, A.G. (2000). Association of GCN1/GCN20 regulatory complex with the conserved N-terminal domain of eIF2α kinase GCN2 is required for GCN2 activation in vivo. EMBO J. *19*, 1887–1899.

Harding, H.P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature *397*, 271–274.

Hatfield, D., Matthews, C.R., and Rice, M. (1979). Aminoacyl-transfer RNA populations in mammalian cells chromatographic profiles and patterns of codon recognition. Biochim. Biophys. Acta *564*, 414–423.

Hinnebusch, A.G. (1996). Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2. In Translational Control, J.W.B. Hershey, M.B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 199–244.

Olsen, D.S., Jordan, B., Chen, D., Wek, R.C., and Cavener, D.R. (1998). Isolation of the gene encoding the *Drosophila melanogaster* homolog of the *Saccharomyces cerevisiae GCN2* eIF-2α kinase. Genetics *149*, 1495–1509.

Pavitt, G.D., Yang, W., and Hinnebusch, A.G. (1997). Homologous segments in three subunits of the guanine nucleotide exchange factor eIF2B mediate translational regulation by phosphorylation of eIF2. Mol. Cell. Biol. *17*, 1298–1313.

Qiu, H., Garcia-Barrio, M.T., and Hinnebusch, A.G. (1998). Dimerization by translation initiation factor 2 kinase GCN2 is mediated by interactions in the C-terminal ribosome-binding region and the protein kinase domain. Mol. Cell. Biol. *18*, 2697–2711.

Ramirez, M., Wek, R.C., and Hinnebusch, A.G. (1991). Ribosome-association of GCN2 protein kinase, a translational activator of the *GCN4* gene of *Saccharomyces cerevisae*. Mol. Cell. Biol. *11*, 3027–3036.

Ramirez, M., Wek, R.C., Vazquez de Aldana, C.R., Jackson, B.M., Freeman, B., and Hinnebusch, A.G. (1992). Mutations activating the yeast eIF-2α kinase GCN2: isolation of alleles altering the domain related to histidyl-tRNA synthetases. Mol. Cell. Biol. *12*, 5801–5815.

Romano, P.R., Garcia-Barrio, M.T., Zhang, X., Wang, Q., Taylor, D.R., Zhang, F., Herring, C., Mathews, M.B., Qin, J., and Hinnebusch, A.G. (1997). Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2α kinases PKR and GCN2. Mol. Cell. Biol. *18*, 2282–2297.

Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J.C., and Moras, D. (1991). Class II aminoacyl transfer RNA synthetases: crystal structure of yeast aspartyl-tRNA synthetase complexed with $tRNA^{Asp}$. Science *252*, 1682–1689.

Santoyo, J., Alcalde, J., Mendez, R., Pulido, D., and de Haro, C. (1997). Cloning and characterization of a cDNA encoding a protein synthesis initiation factor-2α (eIF-2α) kinase from *Drosophila melanogaster*. J. Biol. Chem. *272*, 12544–12550.

Sattlegger, E., Hinnebusch, A.G., and Barthelmess, I.B. (1998). *cpc-3*, the *Neurospora crassa* homologue of yeast *GCN2,* encodes a polypeptide with juxtaposed eIF2α kinase and histidyl-tRNA synthetase-related domains required for general amino acid control. J. Biol. Chem. *273*, 20404–20416.

Schimmel, P.R., and Soll, D. (1979). Aminoacyl-tRNA synthetases: general features and recognition of transfer RNAs. Annu. Rev. Biochem. *48*, 601–648.

Schmedt, C., Green, S.R., Manche, L., Taylor, D.R., Ma, Y., and Mathews, M.B. (1995). Functional characterization of the RNA-binding domain and motif of the double-stranded RNA-dependent protein kinase DAI (PKR). J. Mol. Biol. *249*, 29–44.

Seong, B.L. and RajBhandary, U.L. (1987). *Escherichia coli* formylmethionine tRNA: mutations in GGGCCC sequence conserved in anticodon stem of initiator tRNAs affect initiation of protein synthesis and conformation of anticodon loop. Proc. Natl. Acad. Sci. USA *84*, 334–338.

Shi, Y., Vattem, K.M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R.C. (1998). Identification and characterization of pancreatic eukaryotic initiation factor 2 α-subunit kinase, PEK, involved in translational control. Mol. Cell. Biol. *18*, 7499–7509.

Simos, G., Segref, A., Fasiolo, F., Hellmuth, K., Shevchenko, A., Mann, M., and Hurt, E.C. (1996). The yeast protein Arc1p binds to tRNA and functions as a cofactor for the methionyl- and glutamyl-tRNA synthetases. EMBO J. *15*, 5437–5448.

Sood, R., Porter, A.C., Olsen, D., Cavener, D.R., and Wek, R.C. (2000). A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α. Genetics *154*, 787–801.

Wek, R.C., Jackson, B.M., and Hinnebusch, A.G. (1989). Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling *GCN4* expression to amino acid availability. Proc. Natl. Acad. Sci. USA *86*, 4579–4583.

Wek, R.C., Ramirez, M., Jackson, B.M., and Hinnebusch, A.G. (1990). Identification of positive-acting domains in GCN2 protein kinase required for translational activation of *GCN4* expression. Mol. Cell. Biol. *10*, 2820–2831.

Wek, S.A., Zhu, S., and Wek, R.C. (1995). The histidyl-tRNA synthetase-related sequence in the eIF-2α protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol. Cell. Biol. *15*, 4497–4506.

Zhu, S., Sobolev, A.Y., and Wek, R.C. (1996). Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate *in vitro* phosphorylation of eIF-2. J. Biol. Chem. *271*, 24989–24994.

Zhu, S., and Wek, R.C. (1998). Ribosome-binding domain of eukaryotic initiation factor-2 kinase GCN2 facilitates translation control. J. Biol. Chem. *273*, 1808–1814.