Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2

Hongfang Qiu, Cuihua Hu, Jinsheng Dong, and Alan G. Hinnebusch

Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, Bethesda, Maryland 20892, USA

The protein kinase GCN2 is activated in amino acid-starved cells on binding of uncharged tRNA to a histidyl-tRNA synthetase (HisRS)-related domain. We isolated two point mutations in the protein kinase (PK) domain, R794G and F842L, that permit strong kinase activity in the absence of tRNA binding. These mutations also bypass the requirement for ribosome binding, dimerization, and association with the GCN1/GCN20 regulatory complex, suggesting that all of these functions facilitate tRNA binding to wild-type GCN2. While the isolated wild-type PK domain was completely inert, the mutant PK was highly active in vivo and in vitro. These results identify an inhibitory structure intrinsic to the PK domain that must be overcome on tRNA binding by interactions with a regulatory region, most likely the N terminus of the HisRS segment. As Arg 794 and Phe 842 are predicted to lie close to one another and to the active site, they may participate directly in misaligning active site residues. Autophosphorylation of the activation loop was stimulated by R794G and F842L, and the autophosphorylation sites remained critical for GCN2 function in the presence of these mutations. Our results imply a two-step activation mechanism involving distinct conformational changes in the PK domain.

[Key Words: eIF2α kinase; GCN2; regulation; translation; tRNA]
Figure 1. Phenotypes of GCN2 mutations R794G and F842L. (A) The GCN2 polypeptide of 1659 residues is depicted schematically and divided into the following functional domains: the GCN1/GCN20 binding region [GCN1/20], a degenerate kinase domain [PK], and the C-terminal region, or C-term, required for ribosome-binding and dimerization [RB/DD]. The tRNA-binding domain is comprised of the HisRS and C-term segments. Locations of activating mutations (GCN2c) are indicated above, whereas selected gcn2− mutations are indicated below the schematic. Segments involved in domain interactions are designated by open boxes connected by arrows. (B) Transformants of gcn2Δ strain H1149 carrying empty vector or low-copy plasmids p299 containing gcn2-m2, p722 containing wild-type GCN2, pHQ1096 containing GCN2c-R794G-m2 (R794G-m2), or pHQ1098 containing GCN2c-F842L-m2 (F842L-m2) were replica-plated to SD, SD plus 30 mM 3-AT, or SD plus 0.5 mM 5-FT and 0.125 mM TRA (5FT/TRA) and incubated for 3 days at 30°C. (C) Transformants carrying empty vector, p722, pHQ1102 containing GCN2c-R794G (R794G), pHQ1103 containing GCN2c-F842L (F842L), pHQ1096, or pHQ1098 were streaked on an SC-Ura plate and incubated for 3 days at 30°C. (D) Transformants of gcn2Δ GCD2-K627T strain HQY346 carrying empty vector (gcn2Δ), p722, pHQ1102, pHQ1103, or pHQ1127 containing GCN2Hyper (R794G F842L), were grown in SC-Ura-His medium overnight to saturation, diluted into fresh medium at OD600 = ∼0.2, and grown for 6 h at 30°C (odd-numbered lanes). For histidine starvation, 3-AT was added at 10 mM for 1 h before harvesting (even-numbered lanes). WCEs containing 10 µg of protein were resolved by SDS-PAGE on a 4%–12% NuPAGE gel [Novex] and subjected to Western analysis using antibodies specific for eIF2α phosphorylated on Ser 51 (P-eIF2α, upper panel), or with eIF2α antibodies [lower panel]. The signals on the upper panel were normalized for the corresponding signals in the lower panel, and the ratios of the normalized signals observed in the presence and absence of 3-AT are indicated [Ratio*].
The isolated C-term domain can physically interact with the HisRS and PK domains [summarized in Fig. 1A]. Interestingly, the C-term–PK interaction was weakened by the GCN2<sup>-E803V</sup> mutation in the PK domain [Fig. 1A], which confers constitutive activation of GCN2 function in vivo (Qiu et al. 2001). The fact that <em>E803V</em> also increased the affinity of GCN2 for tRNA implied that tRNA binding is impeded by the PK–C-term interaction, such that tRNA binding would normally occur only in starved cells when uncharged tRNA accumulates. The C-term–PK interaction might inhibit PK activity as well, in which case bound tRNA would dissolve this autoinhibitory interaction. The N-terminal portion of the HisRS region [HisRS-N] also interacts with the PK domain [Fig. 1A; Qiu et al. 2001], and the HisRS-N segment stimulates kinase function independently of its role in tRNA binding or dimerization. Thus, it appears that the HisRS-N region remains engaged with the PK domain in the tRNA-bound state to promote kinase activation.

Here we present compelling evidence that the PK domain itself contains an autoinhibitory function. We isolated two mutations in the PK domain (<em>R794G</em> and <em>F842L</em>) as intragenic suppressors of the tRNA binding motifs for kinase activity.

Previously described GCN2<sup>-</sup> products require the m2 motif for kinase activity

Point mutations in motif 2 of the HisRS-like domain in GCN2 (<em>gcn2-m2</em>, Fig. 1A) abolish tRNA binding in vitro and inactive GCN2 function in vivo [Wek et al. 1995]. Numerous mutations that activate GCN2 constitutively [<em>GCN2<sup>-</sup> alleles</em>] have been isolated in the PK, HisRS-like, and C-term domains [Fig. 1A; Wek et al. 1990; Ramirez et al. 1992]. To determine whether these GCN2<sup>-</sup> products require tRNA binding for activation, we combined the m2 substitutions with each of the plasmid-borne <em>GCN2<sup>-</sup> alleles</em> shown in Figure 1A and analyzed the resulting constructs in a <em>gcn2Δ</em> strain. GCN2<sup>-</sup> function was assessed by measuring growth on medium containing 3-amino-triazole [3AT], an inhibitor of histidine biosynthesis, or on medium containing the amino acid analogs 5-fluorotryptophan [5FT] and 1,2,4-triazolealanine [TRA] [Ramirez et al. 1992]. Growth on 3AT medium requires GCN2-dependent derepression of GCN4 and its target genes in the histidine biosynthetic pathway. GCN2<sup>-</sup> strains are more resistant than wild-type to 5FT and TRA because they produce constitutively derepressed levels of tryptophan and histidine biosynthetic enzymes [Ramirez et al. 1992].

None of the GCN2<sup>-m2</sup> mutants grew on 5FT/TRA medium and only one (<em>GCN2<sup>-M788V-m2</sup></em>) grew weakly on 3AT medium compared to wild type [data not shown]. Hence, the constitutive activation of all previously described GCN2<sup>-</sup> proteins is strongly dependent on tRNA binding. This conclusion was confirmed for the GCN2<sup>-M788V</sup> allele by measuring expression of a GCN2-dependent HIS4-lacZ reporter. The GCN2<sup>-M788V-m2</sup> strain showed repressed levels of HIS4-lacZ in nonstarved cells, comparable to that seen in <em>GCN2<sup>-</sup>, gcn2-m2</em>, or <em>gcn2Δ</em> strains, whereas the parental GCN2<sup>-M788V</sup> strain had fourfold greater HIS4-lacZ activity (Table 1, rows 1–5).

Mutations R794G and F842L in the PK domain constitutively activate the gcn2-m2 product

We set out to isolate a new class of GCN2<sup>-</sup> mutations that would activate GCN2 independently of tRNA binding. As described in Materials and Methods, we identified the <em>R794G</em> and <em>F842L</em> mutations in the kinase domain as strong suppressors of the 3AT-sensitive [3AT<sup>-</sup>] phenotype of <em>gcn2-m2</em>. Both the GCN2<sup>-R794G-m2</sup> and GCN2<sup>-F842L-m2</sup> alleles also conferred resistance to 5FT/TRA [Fig. 1B] and derepression of HIS4-lacZ under nonstarvation conditions compared to GCN2 and <em>gcn2-m2</em> [Table 1, rows 2, 3, 6, and 7]. They also produced a slow-growth [Slg<sup>-</sup>] phenotype on complete (SC) medium [Fig. 1C], characteristic of highly activated GCN2<sup>-</sup> alleles [Ramirez et al. 1992]. Removing the m2 mutations exacerbated both the Slg<sup>-</sup> phenotype [Fig. 1C] and derepression of HIS4-lacZ by R794G and F842L [Table 1, rows 6–9]. Thus, although these mutations greatly reduce the requirement for tRNA binding, restoring motif 2 produced even greater kinase activity.

The allele containing both activating mutations plus m2 conferred a more severe growth defect and greater derepression of HIS4-lacZ than did the GCN2<sup>-R794G-m2</sup> and GCN2<sup>-F842L-m2</sup> alleles [Table 1, rows 6–7, 11], showing that <em>R794G</em> and <em>F842L</em> have cumulative activating effects. Indeed, combining <em>F842L</em> and <em>R794G</em> in the same allele with wild-type motif 2 produced a lethal phenotype [Table 1, row 10]. The latter can be attributed to an intolerably high level of eIF2α phosphorylation because GCN2<sup>-R794G-F842L</sup> had no phenotype in a strain expressing nonphosphorylatable eIF2α (alanine at position 51; data not shown). Henceforth, we refer to GCN2<sup>-R794G-F842L</sup> as GCN2<sup>hyper</sup> [for hyperactivated GCN2].

We confirmed that the activating mutations produce high-level phosphorylation of eIF2α under nonstarvation conditions. Because GCN2<sup>hyper</sup> is lethal in the presence of wild-type eIF2α, we analyzed a mutant strain where eIF2B is resistant to inhibition by phosphorylated eIF2 because of a mutation in its β-subunit [GCD2-K627T; Pavitt et al. 1997]. Using antibodies that recognize eIF2α phosphorylated on Ser 51 [p-eIF2α] to probe whole cell extracts [WCEs], we found that eIF2α was phosphorylated in the GCN2<sup>-R794G</sup>, GCN2<sup>-F842L</sup>, and GCN2<sup>hyper</sup>
strains under nonstarvation conditions, whereas wild-type cells contained an appreciable amount of P-eIF2α only under starvation conditions [Fig. 1D].

The GCN2Hyper PK domain is active in the absence of flanking regulatory domains

We wished to determine whether the GCN2Hyper mutations overcome the requirement for the entire HisRS domain, as well as the ribosome-binding and dimerization functions of the C-term and the GCN1/GCN20 binding domain in the N terminus. To this end, we combined the GCN2Hyper mutations with deletions that remove different flanking segments flanking the PK domain, culminating in a construct that contains only the core PK moiety (Fig. 2). The deletions in the constructs marked with asterisks [rows 3, 5, 6, and 8] destroyed GCN2 function when examined in the absence of the GCN2Hyper mutations, conferring 3AT-sensitivity in a gcn2Δ strain even on high-copy plasmids [data not shown]. Remarkably, none of the deletions in Figure 2 inactivated kinase function when combined with the GCN2Hyper mutations. The last two alleles complemented the 3AT3 phenotype, and derepressed HIS4-lacZ expression above the wild-type level, only when introduced into the gcn2Δ strain on high-copy plasmids [Fig. 2]. Nevertheless, the functionality of the last construct provides a striking demonstration that the GCN2Hyper mutations bypass the need for all regions flanking the PK domain for substantial kinase activity in vivo.

The products of the last three alleles shown in Figure 2 lack the C-term and, thus, should be defective for ribosome binding. To show that the GCN2Hyper mutations did not activate a cryptic ribosome binding activity in the PK domain, we confirmed that GCN2HyperΔ1536–1659 [row 6] and gcn2Δ–Δ1536–1659 were equally defective for ribosome binding in WCEs [data not shown]. Deletion of the C-term from GCN2Hyper also should abolish dimerization. However, we showed previously that the PK domain can dimerize more efficiently when all flanking regions are eliminated, allowing stable PK-PK dimers to form in vivo (Qiu et al. 1998). To determine whether R794G and F842L increase kinase activity by stimulating dimerization of the PK domain, we asked whether these mutations increase the formation of heterodimers by LexA- and HA-tagged PK domains coexpressed in the same cells. As shown in Figure 3A [left panel], ∼15% of a LexA fusion containing the C-terminal two-thirds of the kinase domain [LexA-PK[720–999]] was specifically coimmunoprecipitated with the HA-tagged PK domain [3xHA-PK[591–1010]]. Importantly, the presence of the GCN2Hyper mutations in the LexA- and HA-tagged PK domains coexpressed in the same cells. As shown in Figure 3A [left panel], ∼15% of a LexA fusion containing the C-terminal two-thirds of the kinase domain [LexA-PK[720–999]] was specifically coimmunoprecipitated with the HA-tagged PK domain [3xHA-PK[591–1010]]. Importantly, the presence of the GCN2Hyper mutations in the LexA- and HA-tagged PK domain had no significant effect on the yield of heterodimers [Fig. 3A, right panel]. The same conclusion was reached for analogous constructs containing larger PK segments encompassing residues 591–1010 [data not shown]. We conclude that the GCN2Hyper mutations do not increase kinase activity by stimulating dimerization of the PK domain.

Deleting the GCN1/GCN20 binding domain at the N terminus of GCN2Hyper did not eliminate its lethal phenotype [Fig. 2, row 4], suggesting that the GCN2Hyper...
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Figure 2. All regions flanking the kinase domain are dispensable for the in vivo kinase function of GCN2Hyper. The GCN2 alleles are depicted schematically next to the names of low-copy plasmids on which they were introduced into yeast. Deletions are designated by dashed lines, with the amino acid positions of the first and last deleted residues indicated. Transformants FL-PKHyper (PKHyper) is active in vitro (data not shown). Consistently, phosphorylated eIF2α–AC was readily detected using only 5 nM FL-PKHyper, whereas no P-eIF2α–AC was detected in reactions containing 80-fold more FL-PK (Fig. 3C, lanes 13–15). Thus, the wild-type PK domain (FL-PK) is completely inactive, whereas FL-PKHyper has high-level kinase activity in vivo.

To demonstrate this last point in vitro, we purified the FLAG-tagged proteins and conducted kinase assays using a recombinant form of eIF2α [eIF2α–ΔC] and [γ32P]ATP as substrates. Phosphorylation of eIF2α–AC was readily detected by 5 nM FL-PKHyper, whereas no P-eIF2α–ΔC was detected in reactions containing 80-fold more FL-PK (Fig. 3C, lanes 1–8). These results prove that the wild-type PK domain is intrinsically defective for eIF2α phosphorylation and that the R794G and F842L mutations overcome this defect in kinase function. We showed previously that the E803V mutation in the PK domain leads to constitutive phosphorylation of eIF2α in vivo and increases the tRNA binding activity of GCN2 (Dong et al. 2000). As indicated above, GCN2–E803V was inactivated by the m2 mutation, suggesting that E803V activates GCN2 by increasing its affinity for tRNA rather than eliminating an inhibitory structure in the PK domain. Consistently, purified FL-PKHyperE803V had barely detectable eIF2α kinase activity, at least 100 times lower than that of FL-PKHyper (Fig. 3C, lanes 13–15 vs. 10–12).

Autophosphorylation sites in the activation loop are required for GCN2Hyper activity

GCN2 autophosphorylates in vitro on two threonine residues in the activation loop of the PK domain, Thr 882 and Thr 887. Alanine substitutions at these positions
The core protein kinase domain containing the GCN2Hyper mutations (PKHyper) is active in vivo and in vitro. (A) The GCN2Hyper mutations do not stimulate dimerization of the PK domain. Transformants of strain HQY132 containing plasmid pHQ1241 encoding 3xHA-PK(591–1010) and either pEG202 (lanes 1–3) or pHQ433 (lanes 4–9) encoding, respectively, LexA alone and LexA-PK(720–999), or containing pHQ1222 encoding 3xHA-PK(591–1010)Hyper and either pEG202 (lanes 10–12) or pHQ1235 (lanes 13–18), encoding LexA-PK(720–999)Hyper, were grown in SC-Ura-His medium to OD600 = 1.2, and WCEs were immunoprecipitated with HA antibodies. The immune complexes were subjected to Western analysis with antibodies against LexA [upper panels] or HA [lower panels]. Lanes labeled I, P, or S contain, respectively, 10% of the input WCE, 50% of pellet, and 10% of the supernatant. Lanes 4–6 and 7–9 depict the results of duplicate experiments, as do lanes 13–15 and 16–18. (B) FL-PKHyper phosphorylates eIF2α in vivo. Transformants of HQY346 containing empty vector pEMBL Lex4 [gcn2Δ], pHQ1213 [FL-PKHyper], pHQ1223 [FL-PK], pHQ1242 [FL-PKHyper-K628R], or pDH103 [FL-GCN2] were grown in SC-Ura-His to saturation, diluted into SCGal-Ura-His at OD600 = ~0.2 and grown for 7 h at 30°C [-3-AT, odd-numbered lanes], or for 6 h as just described after which 10 mM 3-AT was added for 1 h [+3-AT, even-numbered lanes]. WCEs were subjected to Western analysis as described in Figure 1D, except that anti-Flag monoclonal antibodies (Sigma) were used to detect the Flag-tagged proteins. (C) PKHyper phosphorylates eIF2α in vitro. The indicated Flag-tagged core PK proteins were expressed in transformants of strain HQY346 harboring plasmids pHQ1213 [FL-PKHyper], pHQ1223 [FL-PK], or pHQ1261 [FL-PKΔE803V], grown in minimal galactose medium and purified with anti-Flag affinity resin. The purified proteins were analyzed for kinase activity, at the protein concentrations indicated across the top of each panel, by incubating with 25 µM [γ-32P]ATP (6000 Ci/mmol, Amersham) and 2.5 µM recombinant eIF2α−H9004 purified from E. coli in 20 µL kinase buffer for 20 min at 30°C. Reactions were stopped by adding 4× SDS-PAGE sample buffer and boiling, resolved by SDS-PAGE, stained with Coomassie blue [lower panel], and the dried gel was subjected to autoradiography [upper panel]. By varying the concentration of eIF2α in the assays (data not shown), we verified that saturating amounts of substrate were employed in these assays.

Figure 3. The core protein kinase domain containing the GCN2Hyper mutations (PKHyper) is active in vivo and in vitro. (A) The GCN2Hyper mutations do not stimulate dimerization of the PK domain. Transformants of strain HQY132 containing plasmid pHQ1241 encoding 3xHA-PK(591–1010) and either pEG202 (lanes 1–3) or pHQ433 (lanes 4–9) encoding, respectively, LexA alone and LexA-PK(720–999), or containing pHQ1222 encoding 3xHA-PK(591–1010)Hyper and either pEG202 (lanes 10–12) or pHQ1235 (lanes 13–18), encoding LexA-PK(720–999)Hyper, were grown in SC-Ura-His medium to OD600 = 1.2, and WCEs were immunoprecipitated with HA antibodies. The immune complexes were subjected to Western analysis with antibodies against LexA [upper panels] or HA [lower panels]. Lanes labeled I, P, or S contain, respectively, 10% of the input WCE, 50% of pellet, and 10% of the supernatant. Lanes 4–6 and 7–9 depict the results of duplicate experiments, as do lanes 13–15 and 16–18. (B) FL-PKHyper phosphorylates eIF2α in vivo. Transformants of HQY346 containing empty vector pEMBL Lex4 [gcn2Δ], pHQ1213 [FL-PKHyper], pHQ1223 [FL-PK], pHQ1242 [FL-PKHyper-K628R], or pDH103 [FL-GCN2] were grown in SC-Ura-His to saturation, diluted into SCGal-Ura-His at OD600 = ~0.2 and grown for 7 h at 30°C [-3-AT, odd-numbered lanes], or for 6 h as just described after which 10 mM 3-AT was added for 1 h [+3-AT, even-numbered lanes]. WCEs were subjected to Western analysis as described in Figure 1D, except that anti-Flag monoclonal antibodies (Sigma) were used to detect the Flag-tagged proteins. (C) PKHyper phosphorylates eIF2α in vitro. The indicated Flag-tagged core PK proteins were expressed in transformants of strain HQY346 harboring plasmids pHQ1213 [FL-PKHyper], pHQ1223 [FL-PK], or pHQ1261 [FL-PKΔE803V], grown in minimal galactose medium and purified with anti-Flag affinity resin. The purified proteins were analyzed for kinase activity, at the protein concentrations indicated across the top of each panel, by incubating with 25 µM [γ-32P]ATP (6000 Ci/mmol, Amersham) and 2.5 µM recombinant eIF2α−H9004 purified from E. coli in 20 µL kinase buffer for 20 min at 30°C. Reactions were stopped by adding 4× SDS-PAGE sample buffer and boiling, resolved by SDS-PAGE, stained with Coomassie blue [lower panel], and the dried gel was subjected to autoradiography [upper panel]. By varying the concentration of eIF2α in the assays (data not shown), we verified that saturating amounts of substrate were employed in these assays.

Thus, GCN2Hyper is strongly dependent on both autophosphorylation sites. Consistently, introducing T887A alone or both T882A and T887A into FL-PKHyper eliminated phosphorylation of eIF2α in vivo, whereas T882A alone led to a small reduction in eIF2α phosphorylation (Fig. 4A). The fact that FL-PKHyper-T887A is inactive whereas GCN2Hyper-T887A retains kinase activity in vivo (Table 1, row 15) is consistent with other results in Table 1 and Figure 2, indicating that the flanking domains enhance PK function in the GCN2Hyper protein.
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Figure 4. Kinase activation by the GCN2\textsuperscript{Hyper} mutations is dependent on autophosphorylation of the activation loop. (A) Transformants of HQY346 containing vector pEMBlcyx4 [lanes 1–2], pHQ1213 (FL-PK\textsuperscript{Hyper}), pHQ1247 (FL-PK\textsuperscript{Hyper}-T882A), pHQ1248 (FL-PK\textsuperscript{Hyper}-T877A), or pHQ1249 (FL-PK\textsuperscript{Hyper}-T882A T887A) were analyzed by Western analysis for levels of phosphorylated eIF2α (1×, 2×, and 4×) were subjected to Western analysis using antibodies specific for GCN2 phosphorylated on Thr 882 [Fig. 3B]. (B–C) The GCN2\textsuperscript{Hyper} mutations increase autophosphorylation of Thr 882. (B) The FL-GCN2 and FL-GCN2\textsuperscript{Hyper} [lanes 1–3] and FL-GCN2\textsuperscript{Hyper} [lanes 4–6] proteins were purified as described in Figure 3 using constructs pDH103 and pHQ1131, respectively, and three different relative amounts of each protein [1×, 2×, and 4×] were subjected to Western analysis using antibodies specific for GCN2 phosphorylated on Thr 882 [upper panel] or Flag antibodies [lower panel]. (C) The FL-PK\textsuperscript{Hyper} [lanes 1–3], FL-PK [lanes 4–6], FL-PK\textsuperscript{Hyper}-T882A, T887A [lane 7], and FL-PK\textsuperscript{Hyper}-K628R [lane 8] proteins were purified as described in Figure 3 using constructs pHQ1213, pHQ1223, pHQ1249, and pHQ1242, respectively, and analyzed as in (B).

Discussion

Having found that the activating mutation GCN2\textsuperscript{E803V} weakened interaction between isolated PK and C-term segments [Qiu et al. 2001] and also stimulated tRNA binding by GCN2, we proposed that the PK–C-term interaction interferes with tRNA binding to the HisRS–C-term domains. This would prevent tRNA binding in nonstarved cells where uncharged tRNA concentrations are low. The PK–C-term interaction could also inhibit PK function by an autoinhibitory mechanism that would be released by tRNA binding [Dong et al. 2000]. Our finding here that the GCN2\textsuperscript{E803V-m2} allele was completely nonfunctional indicates that the weakened interaction between the C-term and PK domains produced by E803V is not sufficient for kinase activation, and that tRNA binding is still required. In fact, it is possible that the C-term has no autoinhibitory function and that C-term–PK association merely occludes tRNA binding contacts in the C-term. In this view, E803V simply allows tRNA binding and kinase activation to occur at low concentrations of uncharged tRNA. As all other previously described GCN2\textsuperscript{E803V} alleles were impaired by the m2 mutation, they may activate GCN2 by this same mechanism.

The GCN2\textsuperscript{Hyper} mutations described here provide a critical new element to our model, as they produce constitutive kinase activation in the absence of tRNA binding. When combined in the same polypeptide, the GCN2\textsuperscript{Hyper} mutations allow the core PK domain to function efficiently under conditions where the wild-type PK domain is completely inert. The simplest explanation for this finding is that the wild-type PK domain is intrinsically defective due to an inhibitory structure involving residues Arg 794 and Phe 842. An important implication of this result is that GCN2 cannot be activated merely by dissociating all flanking regulatory regions from the PK domain; otherwise, the wild-type core PK domain would be active. Rather, the inhibitory structure in the PK domain must be overcome by a stimulatory interaction with another part of the protein.

Based on a sequence alignment of the GCN2 PK domain and protein kinase A (PKA), residues Arg 794 and Phe 842 in GCN2 correspond to Gly 126 and Leu 173 in PKA subdomains V and VIb. Gly 126 in PKA is near the end of the extended chain connecting the N- and C-terminal lobes of the kinase domain [just prior to helix D], whereas Leu 173 occurs in the middle of β-strand 7 near the deep cleft between the two lobes [Fig. 5]. As the site of catalysis lies within this cleft, both residues are near the active site. In fact, the residue adjacent to Gly 126 in PKA helps in anchoring ATP and the peptide substrate to...
the active site, while Leu 173 is only two residues away from an invariant residue in the catalytic loop (Asn 171; Hanks and Hunter 1995b). Computer analysis of PKA (using WebLab ViewerLite 3.2) revealed that Gly 126 and Leu 173 are nearly as close to one another in the folded structure as adjacent residues in the polypeptide chain (Fig. 5). Based on these considerations, we predict that Arg 794 and Phe 842 play a direct role in distorting the GCN2 active site. Consistently, the $GCN2^{Hyper}$ mutations replace these residues with the exact amino acids (Gly and Leu, respectively) found at the equivalent positions in PKA, an intrinsically active kinase, and in a majority of other protein kinases (Hanks and Hunter 1995a).

The HisRS-N region is required for activation of GCN2 independently of its role in tRNA binding. The HisRS-N segment binds to the PK domain between residues 750 and 810 (Qiu et al. 2001), encompassing the predicted extended connector between the N- and C-lobes of the PK domain and including the inhibitory residue Arg 794. Thus, an attractive hypothesis is that association of the HisRS-N/tRNA complex with this surface of the PK domain alters the relative orientation of the kinase lobes in a way that overcomes the misalignment of active site groups imposed by wild-type residues Arg 794 and Phe 842.

Phosphorylation of the activation loop is frequently required for kinase activation. It is thought that the phosphorylated residue stabilizes a positively charged cluster and thereby promotes the correct orientation and electrostatic environment of the catalytic aspartate (Asp 166 in PKA, Fig. 5) and the proper relative orientation of the N- and C-lobes (Johnson et al. 1996). We found that the $GCN2^{Hyper}$ mutations did not bypass the requirement for the autophosphorylation sites in the GCN2 activation loop, as replacement of Thr 887 and Thr 882 with alanine almost completely inactivated $GCN2^{Hyper}$. Replacement of Thr 887 alone was sufficient to inactivate the core kinase domain in FL-PK$^{Hyper}$. Based on these findings, we propose that phosphorylation of the activation loop occurs only after the inhibitory structure of the PK domain has been corrected by interaction with the HisRS-N/tRNA module. Consistently, Thr 882 was phosphorylated in the highly active FL-PK$^{Hyper}$ protein but not in the inactive wild-type core PK construct. Presumably, autophosphorylation stimulates a critical conformational change in the PK domain beyond that produced by association with the HisRS-N/tRNA module. This model has similarities with a mechanism proposed for activation of CDK2 by cyclin, in which binding of cyclin A realigns the active site residues and relieves the steric block to substrate binding imposed by the PSTAIRE helix and activation loop. Phosphorylation of the activation loop is additionally required for full activation of CDK2 (Jeffrey et al. 1995; Johnson et al. 1996).

The fact that the $GCN2^{Hyper}$ mutations bypass the requirement for the N-terminal GCN1/GCN20 binding domain and the C-term, in addition to the HisRS domain, suggests that all of these domains are required for activation of GCN2 by uncharged tRNA. This conclusion supports our proposal that the GCN1/GCN20 complex interacts with the N terminus of GCN2, anchored to the ribosome through the C-term, to mediate transfer of uncharged tRNAs from the decoding site on the ribosome to the HisRS domain (Sattlegger and Hinnebusch 2000).

Materials and methods

Isolation of GCN2$^{R794G}$ and GCN2$^{F842L}$

GCN2 plasmid pHQ644 was subjected to random mutagenesis in E. coli mutator strain XL1-Red [Stratagene]. BspEI–Asp718

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fragments were isolated from the mutagenized pHQ644 and used to replace the corresponding fragment of plasmid p299 containing the gcn2-m2 allele (library II). Similarly, the EcoRI–NheI fragment of plasmid p299 was replaced with the corresponding fragments from mutagenized pHQ644 to produce library II. The two libraries were screened in gen2Δ strain H1149 for 3-ATT transformants. Of 3.4 × 10⁵ transformants obtained, 882 (TTT to CTT), generating F842L.

All other plasmids used in this work are listed in Table 2, and details of their construction are available on request. Strains H1149 [Wek et al. 1990] and HQY1132 [Qiu et al. 1998] were described previously. Strain HQY346 is a GAL derivative of GP5299 [Pavitt et al. 1997].

**Biochemical methods**

Transformants of HQY346 bearing the appropriate plasmids encoding Flag-, 6xHis-tagged GCN2 proteins were grown in SD medium to saturation, diluted into minimal medium with 2% galactose (SGal) and grown to OD₆₀₀ = 3.0. Affinity purification of the tagged proteins was conducted essentially as described [Dong et al. 2000]. In vitro kinase assays [Dong et al. 2000] and assays of HIS₄-lacZ expression [Lucchini et al. 1984] were all done as described previously. The enhanced chemiluminescence (ECL) system [Amersham Pharmacia Biotech] was used to detect immune complexes, and Western signals were quantified by video image densitometry using NIH Image 1.61 software. Phosphospecific antibodies against eIF2α [Biosource International] and polyclonal antibodies against eIF2α [CM-217] [Cigan et al. 1989], antibodies against GCN2 [Romano et al. 1998], and antibodies against LexA [Qiu et al. 1998] were described previously. Flag- or HA-tagged proteins were detected using FLAG (Sigma) and HA antibodies (Roche). Antibodies specific for GCN2 phosphorylated on Thr 882 were produced by Quality Controlled Biochemicals against a synthetic peptide corresponding to the GCN2 activation loop phosphorylated on Thr 882 [in single-letter code: CPGSSDNL[P-T882]SAI]. The antibodies were preabsorbed with the nonphosphorylated peptide and affinity-purified with the phosphorylated peptide. In Western blot analysis, the antibodies reacted with FL-GCN2 (wild type) but not with FL-GCN2-T882A or catalytically inactive FL-GCN2-K628R.

**Table 2. Plasmids used in this study**

| Plasmid | Allele | Reference |
|---------|--------|-----------|
| p722    | GCN2, CEN6, URA3 | Wek et al. 1990 |
| p299    | gcna-m2 in p722 backbone | Wek et al. 1995 |
| p912    | GCN2-M788V in p722 backbone | Ramirez et al. 1992 |
| pHQ928  | GCN2-R794g-m2 in p722 backbone | This study |
| pHQ1096 | GCN2-R794g in pHQ644 backbone | This study |
| pHQ1098 | GCN2-F842L-m2 in pHQ644 backbone | This study |
| pHQ1102 | GCN2-F842L in pHQ644 backbone | This study |
| pHQ1103 | GCN2-F842L in pHQ644 backbone | This study |
| pHQ1107 | GCN2-f25p in pHQ644 backbone | This study |
| pHQ1110 | GCN2-f25 in pHQ644 backbone | This study |
| pHQ1111 | high copy Flag-6xHis-GCN2f25 under GAL promoter in pEMBLyex4 | This study |
| pHQ1112 | GCN2-f25p in pHQ644 backbone | This study |
| pHQ1139 | GCN2f25p-Δ536-1659 in pHQ644 backbone | This study |
| pHQ1140 | GCN2f25p-m2 in pHQ644 backbone | This study |
| pHQ1141 | GCN2f25p-Δ110-235 in pHQ644 backbone | This study |
| pHQ1165 | GCN2f25p-Δ3-567 in pHQ644 backbone | This study |
| pHQ1170 | GCN2f25p-Δ1247-1402 in pHQ644 backbone | This study |
| pHQ1193 | GCN2f25p-Δ1011-1659 in pHQ644 backbone | This study |
| pHQ1211 | high copy GCN2f25p-Δ1011-1659 in YEplac195 | This study |
| pHQ1213 | high copy Flag-6xHis-PK(591-1010)f25p under GAL promoter in pEMBLyex4 | This study |
| pHQ1220 | Flag-6xHis-PK(591-1010)f25p in p722 backbone | This study |
| pHQ1222 | high copy 3xHA-PK(591-1010)f25p in p630 backbone | This study |
| pHQ1223 | high copy Flag-6xHis-PK(591-1010)f25p under GAL promoter in pEMBLyex4 | This study |
| pHQ1235 | high copy LexA-ΔPK(720-999)f25p in pEG202 backbone | This study |
| pHQ1241 | high copy 3xHA-PK(591-1010) in p630 backbone | This study |
| pHQ1242 | high copy Flag-6xHis-PK(591-1010)f25p-K628R under GAL promoter in pEMBLyex4 | This study |
| pHQ1247 | high copy Flag-6xHis-PK(591-1010)f25p-T882A under GAL promoter in pEMBLyex4 | This study |
| pHQ1248 | high copy Flag-6xHis-PK(591-1010)f25p-T882A under GAL promoter in pEMBLyex4 | This study |
| pHQ1249 | high copy Flag-6xHis-PK(591-1010)f25p-T882A and T887A under GAL promoter in pEMBLyex4 | This study |
| pHQ1251 | GCN2f25p-T882A in pHQ644 backbone | This study |
| HQP1252 | GCN2f25p-T882A in pHQ644 backbone | This study |
| pHQ1253 | GCN2f25p-T882A in pHQ644 backbone | This study |
| pHQ1261 | high copy Flag-6xHis-PK(591-1010)f25p under GAL promoter in pEMBLyex4 | This study |
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Hongfang Qiu, Cuihua Hu, Jinsheng Dong, et al.

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