Histidyl-tRNA Synthetase-related Sequences in GCN2 Protein Kinase Regulate in Vitro Phosphorylation of eIF-2*

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In yeast, starvation for amino acids stimulates GCN2 phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2). Phosphorylation of eIF-2α induces the translational expression of GCN4, a transcriptional activator of the general amino acid control pathway. It has been proposed that GCN2 sequences containing homology to histidyl-tRNA synthetases (HisRS) bind uncharged tRNA that accumulate during amino acid limitation and stimulate the activity of GCN2 kinase. In this report we address whether the HisRS-related sequences are required for GCN2 phosphorylation of eIF-2α in an in vitro assay. To measure the activity of GCN2 kinase in cellular extracts, we expressed and purified a truncated form of yeast eIF-2α. Phosphorylation of the recombinant eIF-2α substrate was dependent on both GCN2 kinase activity and the eIF-2α phosphorylation site, serine 51. Mutations in the HisRS-related domain of GCN2, which have been shown to block phosphorylation of eIF-2α in vivo and the subsequent stimulation of the general control pathway, also greatly reduced eIF-2α phosphorylation in the in vitro assay. These results indicate that the HisRS-related sequences are required for activation of GCN2 kinase function.

A family of serine/threonine protein kinases regulate protein synthesis by phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2)α (1–4). In mammals, two different protein kinases are known to phosphorylate the regulated site in eIF-2α, serine 51. One is the RNA-dependent protein kinase (PKR), which is a component of the antiviral defense mechanism mediated by interferon (3–5) and is thought to function as a suppressor of cell proliferation and tumorigenesis (6–8). The second protein kinase, heme-regulated inhibitor, is found predominately in reticulocytes and bone marrow and is activated by reduced heme levels or heat shock (9). Phosphorylation of mammalian eIF-2α inhibits general protein synthesis by impairing the conversion of eIF-2-GDP to eIF-2-GTP, a nucleotide exchange that is required for each round of translation initiation (1, 2, 10). In yeast Saccharomyces cerevisiae, a third eIF-2α kinase, GCN2 (general control nonderepressible), participates in the regulation of gene-specific translation (4, 11).

In yeast, starvation for any one of several different amino acids enhances the translation of GCN4, a transcriptional activator of more than 30 genes involved in the biosynthesis of amino acids (12). Control of GCN4 translation involves four short upstream open reading frames located in the 5′-untranslated region of the GCN4 mRNA. In cells not limiting for amino acids, the upstream open reading frames block translational initiation of the GCN4 coding sequences. During amino acid starvation, GCN2 phosphorylation of eIF-2α is thought to lead to lowered eIF-2-GTP levels, thus reducing the inhibitory effects of the upstream open reading frames and allowing for increased GCN4 translation (11, 13, 14).

How does amino acid starvation stimulate the kinase function of GCN2? The carboxyl-terminal portion of the GCN2 protein kinase has homology to the entire sequence of histidyl-tRNA synthetases (HisRS) (Fig. 1) (15). It has been proposed that uncharged tRNA that accumulates in cells starving for amino acids interacts directly with the HisRS-related sequences and stimulates phosphorylation of eIF-2α by GCN2 (14–16). Consistent with this model, we have shown that cells starving for any one of several different amino acids or containing a defective aminoacyl-tRNA synthetase allele, increase eIF-2α phosphorylation by GCN2 (16). Class II aminoacyl-tRNA synthetases, including HisRS (17, 18), share three motifs, and mutations in the motif 2 sequence of GCN2 abolished phosphorylation of eIF-2α during these limiting conditions. This suggests that the HisRS-related sequences measure starvation for many different amino acids by interaction with their cognate uncharged tRNAs. In vitro binding studies showed that recombinant protein containing the HisRS-related domain of GCN2 can bind uncharged tRNA by a process requiring motif 2 sequences (16).

In this report we addressed whether the HisRS-related sequences are required for GCN2 phosphorylation of eIF-2α in an in vitro assay. Previous studies have relied on autophosphorylation of GCN2 in immunoprecipitation complexes as a measure of the activity of GCN2 kinase. To assay for the activity of GCN2 kinase in cellular extracts, we prepared a recombinant substrate containing a portion of yeast eIF-2α sequences. Phosphorylation of this recombinant substrate was dependent on GCN2 kinase activity and the eIF-2α phosphorylation site, serine 51. Mutations in the HisRS-related sequences that blocked stimulation of the general control pathway also greatly reduced in vitro phosphorylation of the recombinant eIF-2α. These results support the role of the HisRS-related sequences in the activation of GCN2 kinase function. Additionally, we observed that amino-terminal sequences in GCN2 that contain homology to a portion of a second kinase domain are required for in vitro phosphorylation of the eIF-2α.

MATERIALS AND METHODS

Yeast Strains and Plasmids—Yeast strain H1149 (MATa gen2::LEU2 ino1 ura3–52 leu2–3 leu2–112 HIS4-lacZ) (15) was transformed with plasmid p630 (19) that encodes GCN2 on the high copy

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The abbreviations used are: eIF, eukaryotic initiation factor; PKR, RNA-dependent protein kinase; HisRS, histidyl-tRNA synthetase(s); PAGE, polyacrylamide gel electrophoresis.
Histidine-rich Sequences Regulate GCN2 Kinase

In Vitro Kinase Assays—Strain H1149 (gen2::LEU2) was transformed with different GCN2 alleles in the histidyl-arginine rich (HAR) leader sequence of eIF-2α. The amino acid content of the HAR sequence is illustrated by the box. In the middle portion of the protein is a domain homologous to protein kinases, and in the carboxyl-terminal portion of GCN2 is a sequence homologous to the entire length of HIS3 (15). This region serves as a stimulus to GCN2 kinase activity by binding to uncharged tRNA that accumulates during amino acid starvation conditions. In this HIS3-related domain there are three motifs, designated m1, m2, and m3, conserved among the class II aminoacyl-tRNA synthetases (16). The gcn2-m2 mutant contains Leu-Leu residues substituted for the invariant Arg residue at positions 1050 and 1051, respectively, in the motif 2 sequence of the HisRS-related domain (15, 31). The gcn2-m2 mutant samples were applied to a column containing nickel chelation resin, and proteins were eluted with 200 mM imidazole in solution A. The molecular weights of the full-length eIF-2α and eIF-2α-ΔC fusion proteins were 38,000 and 28,000, respectively, which are in close agreement with that predicted from the DNA sequence. These recombinant proteins were absent from an identically prepared extract from BL21(DE3) transformed with vector pET-15b. Additional information on the purification of the eIF-2α fusion protein to the nickel chelation resin, samples were concentrated and desalted using a Amicon Centricon concentrator (exclusion size of M, 10,000). The eIF-2α fusion proteins were stored in solution containing 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, and 40% glycerol. Full-length eIF-2α was stored in aliquots at -20°C, and eIF-2α-ΔC was stored at

Results

Mutations in the HisRS-related Sequence Do Not Reduce GCN2 Activity in the Immunoprecipitation Kinase Assay—The
HisRS-related sequences in GCN2 are required for in vivo phosphorylation of eIF-2 and the subsequent stimulation of the general control pathway in response to starvation for amino acids (16). The present investigation sought to determine whether mutations in the HisRS-related domain affect the in vitro kinase activity of GCN2. Previous in vitro studies have assayed GCN2 kinase activity by measuring GCN2 autophosphorylation in immunoprecipitation complexes (19). We immunoprecipitated GCN2 using antiserum prepared against the kinase domain of GCN2, incubated the immune complex with [γ-32P]ATP, and separated in a 7.5% SDS-polyacrylamide gel, and the gel was stained by Coomassie Blue. Radiolabeled proteins were visualized by autoradiography.

Wild-type GCN2 kinase and mutant gcn2-m2 show similar levels of autophosphorylation in the immunoprecipitation kinase assay. Protein extracts were prepared from gcn2::LEU2 strain H1149 transformed with different plasmid-borne GCN2 alleles, and the GCN2 kinase was characterized by immunoblot (A) and immunoprecipitation kinase assays (B). A, GCN2 immunoblot assay using protein extracts prepared from strain H1149 containing the indicated GCN2 allele. B, GCN2 immunoprecipitation kinase assays were carried out using the same extract preparation utilized in the immunoblot experiment. Immunoprecipitated GCN2 was incubated with [γ-32P]ATP and separated in a 7.5% SDS-polyacrylamide gel, and the gel was stained by Coomassie Blue. Radiolabeled proteins were visualized by autoradiography. C, photograph of the Coomassie-stained GCN2 protein from the immunoprecipitation experiment shown in B.

Characterization of gcn2-m2 mutant protein, containing mutations in the motif 2 of the HisRS-related domain, revealed levels of phosphorylation in the immunoprecipitation kinase assay similar to that of wild-type GCN2 (Fig. 2B). This result is consistent with previous work showing that selected GCN2 mutants defective for stimulation of the general control pathway have near wild-type levels of autophosphorylation in the immunoprecipitation assay (19). The fact that wild-type and mutant gcn2-m2 proteins were similarly phosphorylated suggests that autophosphorylation may not be a fully reliable measure of the activity of GCN2 kinase toward exogenous substrates; alternatively, the immunoprecipitation process may have interfered with regulation GCN2 kinase activity.

HisRS-related Sequences of GCN2 Are Required for in Vitro Phosphorylation of Recombinant Yeast eIF-2α—To assay for GCN2 kinase activity using eIF-2α as exogenous substrate, we expressed yeast eIF-2α in E. coli as a polyhistidine fusion protein (see “Materials and Methods”) (Fig. 4A). We first tested the efficacy of the recombinant eIF-2α as a substrate using the related eIF-2α kinase PKR. The eIF-2α was phosphorylated by recombinant murine PKR, and no phosphorylation was detected in the absence of the protein kinase (Fig. 4B). Consistent with phosphorylation of eIF-2α being on serine 51, no phosphorylation was detected in a control assay containing recombinant eIF-2α that had an alanine substituted for serine 51 (Fig. 4B, lane 3). When recombinant eIF-2α was used as the substrate in the GCN2 immunoprecipitation kinase assay, we also found that eIF-2α was phosphorylated (Fig. 4C). However, similar levels of eIF-2α phosphorylation were observed with the gcn2-K559R sample, indicating that phosphorylation of eIF-2α was independent of GCN2 activity. In parallel experiments, we used cellular extracts instead of immunoprecipitated GCN2 and again found similar levels of eIF-2α phosphorylation in samples prepared from isogenic GCN2 and Δgcn2 strains (data not shown).

Previous studies (22, 26), in vivo and in vitro, indicated that casein kinase II phosphorlates residues located in the carboxy-terminal region of yeast eIF-2α. Reasoning that these sites may account for the observed GCN2-independent phosphorylation, we deleted these sequences from the recombinant eIF-2α and produced a truncated form of the protein, eIF-2α-ΔC (Fig. 4A). The eIF-2α-ΔC substrate, which lacked residues 200–304, was phosphorylated by murine PKR (Fig. 4B). We then tested whether the truncated substrate was phosphorylated in a cellular extract containing GCN2. The eIF-2α-ΔC was phosphorylated by extracts from wild-type cells, whereas extracts from isogenic strains with GCN2 deleted (Δgcn2) or containing the kinase-defective allele, gcn2-K559R, showed no phosphorylation of the substrate (Fig. 5). Phosphorylation of eIF-2α-ΔC appeared to be on serine 51, since an identically prepared eIF-2α substrate with alanine substituted for the phosphoryl-
Recombinant yeast eIF-2α is phosphorylated by murine PKR. A, yeast eIF-2α fused to a polyhistidine sequence was expressed and purified in E. coli as described under "Materials and Methods." Purified eIF-2α was analyzed by SDS-PAGE, stained with Coomassie Blue and photographed. The full-length recombinant protein contains the entire eIF-2α sequence from residue 1 to 304, and the truncated version, eIF-2α-ΔC, contains residues 1–199. The wild-type (WT) versions of eIF-2α contain the phosphorylation site serine 51, and mutant versions of eIF-2α contain alanine substituted for serine 51 (S51A). B, murine PKR expressed in E. coli was incubated with [γ-32P]ATP and recombinant eIF-2α, analyzed by SDS-PAGE, and visualized by autoradiography. Reactions analyzed in lanes 1–3 contain lysates from E. coli expressing PKR, and lanes 4 and 5 are control reactions receiving lysates from E. coli cells expressing no PKR. The eIF-2α substrate used in the sample lanes is as follows. Lanes 1 and 4, full-length eIF-2α containing serine 51; lane 2 and 5, eIF-2α-ΔC with serine 51; lane 3, full-length eIF-2α with alanine substituted for serine 51 (S51A). Phosphorylated PKR, full-length eIF-2α, and eIF-2α-ΔC are indicated to the right. Sizes of protein standards in kDa are shown to the left. C, the full-length eIF-2α is phosphorylated in the immunoprecipitation kinase assay independent of GCN2 activity. GCN2 immune complex was incubated with [γ-32P]ATP and full-length eIF-2α protein, and samples were analyzed by SDS-PAGE, followed by autoradiography. Reaction samples 1–3 contain wild-type GCN2, and lanes 4–6 contain the kinase-defective gcn2-K559R. No eIF-2α substrate was added to sample lanes 1 and 4, full-length eIF-2α with serine 51 was added to lanes 2 and 5, and full-length eIF-2α with alanine substituted for serine 51 was added to lanes 3 and 6. Phosphorylated GCN2 and full-length eIF-2α are indicated to the right, and size standards in kDa are shown to the left.

GCN2 kinase assays as well as in cells containing a previously described insertion mutation located in the HisRS-related domain, gcn2–1092EL, that contains Glu-Leu inserted after residue 1092 (Fig. 1). Cells containing this mutation are not able to stimulate the general control pathway in response to histidine starvation (15). Extracts from a gcn2–1092EL mutant strain phosphorylated eIF-2α-ΔC at less than 7% of the levels detected in the wild-type GCN2 sample, and autophosphorylation of gcn2–1092 protein was also reduced (Fig. 6). The level of eIF-2α-ΔC phosphorylation in the gcn2-m2 reaction was less than 10% of that measured in the wild-type GCN2, and the levels in the Δgen2 and gen2-K559R samples were less than 5% of that found in the GCN2 reaction. Sizes of protein standards in kDa are shown to the left.

The level of eIF-2α-ΔC phosphorylation in the gcn2-m2 reaction was less than 10% of that measured in the wild-type GCN2, and the levels in the Δgen2 and gen2-K559R samples were less than 5% of that found in the GCN2 reaction. Sizes of protein standards in kDa are shown to the left.

Deletion of Amino-terminal Sequences Containing Duplicated Kinase Motifs Abolish GCN2 Phosphorylation of eIF-2α Substrate—The amino-terminal portion of GCN2, from residue 320 to 450, contains sequences invariant in subdomains VI–XI of eukaryotic protein kinases (Fig. 1). Curiously, no glycine-rich sequence important for nucleotide binding in kinases is detected in these sequences. Because an in-frame deletion of this region in GCN2 abolished stimulation of the general control
the phosphorylated forms of these truncated GCN2 proteins. To determine if GCN2 activity required the HisRS-related sequences in the absence of amino acid limitation (16), we generated a truncated form of eIF-2 activity in GCN2 mutant cells containing a defective aminoacyl-tRNA synthetase. This increase in eIF-2 phosphorylation appeared to be directly controlled by uncharged tRNA levels because mutant cells containing a defective aminoacyl-tRNA synthetase showed elevated levels of phosphorylation in eIF-2α in the absence of amino acid limitation (16). To determine if GCN2 activity required the HisRS-related sequences in an in vitro assay, we generated a truncated form of eIF-2α, to serve as a kinase substrate in crude yeast lysates. We showed that the eIF-2αΔC substrate was phosphorylated in vitro by a process requiring active GCN2 kinase and the phosphorylation site serine 51. Phosphorylation of eIF-2αΔC was also greatly reduced when GCN2 contained mutations in the HisRS-related sequences. One of the mutants used in this study, gcn2-m2, was found previously to impair in vitro binding with uncharged tRNA (16). We observed, however, that the gcn2-m2 protein could autophosphorylate in the immunoprecipitation kinase assay, which argues against these mutant residues inactivating GCN2 function by simply disrupting the basic structure of the kinase domain. Accumulatively, these in vivo and in vitro studies strongly support the model that the HisRS-related sequences function as a regulatory domain that binds different uncharged tRNA and stimulates the activity of the adjacent GCN2 kinase region.

An important long term goal of our in vitro studies on the regulation of GCN2 is to determine which tRNAs stimulate kinase function. In the studies presented in this report, we have used extracts prepared from cells that were not limited for amino acids. We have carried out parallel experiments using lysates from amino acid-starved cells and have not found any further increase in phosphorylation of eIF-2αΔC by GCN2 kinase (data not shown). Our interpretation of this observation is that activation of GCN2 kinase may occur during the collection and breakage of cells grown under nonstarvation conditions. The cell lysates used in the kinase assays presented in this study contain tRNA, and the charging levels may be substantially reduced in a cell lysate. Additionally, breakage of cells with glass beads may release activating RNA, such as immature tRNA, from cellular compartments not accessible to GCN2 in vivo. In support of this premise, we have added uncharged total yeast tRNA or in vitro synthesized tRNA18s to crude lysates or more enriched preparations of GCN2 and have failed to observe any further enhancement of GCN2 phosphorylation of eIF-2αΔC. Experiments characterizing more purified samples of GCN2 should address this issue, although the apparent requirement for GCN2 association with ribosomes for in vivo phosphorylation of eIF-2αΔC (27) may complicate these studies.

Our analysis of the HisRS-related mutants in the in vitro kinase assay indicated a reduction in both the levels of autophosphorylation and eIF-2α phosphorylation. This coupling implies that GCN2 phosphorylation may participate in the activation of this kinase. A previous study (19) also observed that GCN2 is phosphorylated in cells grown in the presence of [32P]orthophosphate, but the level of radioactivity was not appreciably different between reactions using wild-type GCN2 and kinase-defective gcn2-K559R. This suggests that there may be multiple phosphorylation sites in GCN2, some of which serve as substrates for a second protein kinase. The location and role of phosphorylation in the stimulation of GCN2 kinase

**Fig. 6.** Mutations in the HisRS-related domain and aminoterminal sequences of GCN2 reduce in vitro phosphorylation of eIF-2α. Protein extracts prepared from gcn2::LEU2 strain H1149 transformed with the indicated plasmid-borne GCN2 alleles were mixed with [γ-32P]ATP and eIF-2αΔC containing serine 51. Radiolabeled proteins were separated by 7.5–15% SDS-PAGE, followed by autoradiography. Phosphorylated GCN2 and eIF-2α were indicated by arrows in lanes gcn2-Δ15–42 and gcn2-Δ360–435. Phosphorylation of eIF-2αΔC in the gcn2-Δ15–42 and gcn2-Δ360–435 reactions was less than 5% of the levels measured in the GCN2 sample. Sizes of protein standards in kDa are shown to the left.

**Fig. 7.** Immunoblot assay of mutant and wild-type GCN2 proteins. Protein extracts were prepared from gcn2::LEU2 strain H1149 transformed with the designated plasmid-borne GCN2 alleles and separated by electrophoresis in a 7.5% SDS-polyacrylamide gel. GCN2 protein was visualized by immunoblot analysis.

**DISCUSSION**

The HisRS-related sequences have been proposed to enhance GCN2 phosphorylation of eIF-2α in response to interaction with different uncharged tRNAs that accumulate in cells starving for amino acids (14–16). Prior to this work, the evidence supporting this model included the observation that phosphorylation of eIF-2α by GCN2 in vivo was greatly elevated in response to starvation for one of several different amino acids (14, 16). This increase in eIF-2α phosphorylation appeared to be directly controlled by uncharged tRNA levels because mutant cells containing a defective aminoacyl-tRNA synthetase showed elevated levels of phosphorylation of eIF-2α in the absence of amino acid limitation (16). To determine if GCN2 activity required the HisRS-related sequences in an in vitro assay, we generated a truncated form of eIF-2α, suitable to serve as a kinase substrate in crude yeast lysates. We showed that the eIF-2αΔC substrate was phosphorylated in vitro by a process requiring active GCN2 kinase and the phosphorylation site serine 51. Phosphorylation of eIF-2αΔC was also greatly elevated in the gcn2-Δ15–42 and gcn2-Δ360–435 samples. The level of phosphorylation of eIF-2αΔC in the gcn2-Δ15–42 and gcn2-Δ360–435 reactions was less than 5% of the levels measured in the GCN2 sample. Sizes of protein standards in kDa are shown to the left.
function is not yet understood. It is noteworthy, however, that autophosphorylation is thought to be an obligate step in the activation of PKR, the related eIF-2α kinase that is also regulated by RNA (3, 28, 29). Future studies that identify and mutate phosphorylation sites in GCN2 and PKR will be important to address the role of post-translation modification in the regulation of these eIF-2α kinases.

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