Dimerization Is Required for Activation of eIF2 Kinase Gcn2 in Response to Diverse Environmental Stress Conditions*

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In the yeast Saccharomyces cerevisiae, starvation for amino acids induces phosphorylation of the α subunit of eukaryotic initiation factor 2α by protein kinase Gcn2p (4). The eIF2 complexed with GTP and initiator Met-tRNAMet participates in the ribosomal selection of the start codon (5). During this translation initiation process, the GTP associated with eIF2 is hydrolyzed to GDP, and eIF2 is released from the ribosome. A guanine nucleotide exchange factor, eIF2B, is required to recycle eIF2-GDP to the active eIF2-GTP form (5). Gcn2p phosphorylation of eIF2α at Ser-51 converts this initiation factor from a substrate to an inhibitor of the eIF2B, thereby reducing the levels of eIF2-GTP available for translation initiation (2, 6–8). Lowered levels of eIF2-GTP enhance the translational expression of Gcn4p, a basic zipper transcriptional activator of hundreds of genes important for alleviation of nutrient deprivation (3).

We have been interested in the mechanisms by which Gcn2p is activated during amino acid starvation. Mutations in aminoacyl-tRNA synthetase enzymes, such as HTS1 important for charging of tRNAUaa, elicit the general control response in yeast even in the presence of abundant cognate amino acids (9). This observation supports the idea that increased levels of uncharged tRNA that accumulate during severe amino acid depletion is the direct signal activating the general control pathway. Gcn2p activation by elevated levels of uncharged tRNA involves direct binding of tRNA to a regulatory domain of Gcn2p with sequence homology to the entire length of the HisRS enzymes (9–12). Such binding is thought to be obligatory for Gcn2p activity, as residue substitutions in the HisRS-related domain that impair binding to uncharged tRNA block Gcn2p phosphorylation of eIF2α and promote the general control response (9, 11, 13). Gcn2p binding with uncharged tRNA is not restricted to uncharged tRNAUaa, however, suggesting that the HisRS-related domain has diverged sufficiently from the bona fide HisRS enzyme so that it can bind to many different uncharged tRNA species that amass during amino acid starvation conditions. Gcn2p appears to have reduced affinity for aminoacylated tRNA in vitro, consistent with the idea that it is activated by only uncharged tRNA (11).

Activation of Gcn2p by uncharged tRNA is proposed to involve a transition from an inhibited to a catalytically active conformation that is signaled by direct contacts between the protein kinase domain, HisRS-regulatory region, and the extreme carboxyl terminus of Gcn2p (12, 14, 15). Accompanying this activated Gcn2p conformation is autophosphorylation at threonine residues 882 and 887 in the so-called activation loop (16). This autophosphorylation may occur in trans between Gcn2p dimers. Gcn2p is thought to retain its induced eIF2 kinase activity until it is

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1 The abbreviations used are: eIF2, eukaryotic initiation factor-2; HisRS, histidyl-tRNA synthetase; 3-AT, 3-aminotriazole; ORF, open reading frame; PKR, double-stranded RNA-dependent protein kinase.
Dimerization and Activation of Gcn2

Gcn2p regulation of Gcn2p. Impaired Gcn2p dimerization blocked activation of its different mutants indicate that hydrophobic interactions in the extreme carboxyl terminus of Gcn2p is to facilitate binding of regions of Gcn2p, possibly contributing to the activated and inactivated states of Gcn2p. Two-hybrid and co-immunoprecipitation analyses, the carboxyl terminus of Gcn2p is suggested to interact with multiple regions of Gcn2p, possibly contributing to the activated and inhibitory Gcn2p conformations (14). A final proposed role for the extreme carboxyl terminus of Gcn2p is to facilitate binding of the HisS2-related domain to uncharged tRNA (11). We wished to address the contribution of dimerization to Gcn2p regulation of Gcn4p translation. Our analyses of 16 different mutants indicate that hydrophobic interactions in the carboxyl-terminal region form the dimerization interface of Gcn2p. Impaired Gcn2p dimerization blocked activation of its eIF2 kinase activity and subsequent enhanced Gcn4p expression in response to amino acid limitation or exposure to rapamycin in response to amino acid limitation or exposure to rapamycin.

Gcn2p derivatives encoding wild type or mutant versions of Gcn2p-C were expressed in strains BL21 (DE3) (pET28C, pET28C containing lysogen DE3), and the transformants were grown at 37 °C with shaking in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an A660 between 0.4 and 0.6. 1 mM isopropyl β-D-thiogalactoside was added to the cultures, and after further incubation at 37 °C for 3 h, cells were harvested by centrifugation. The cell pellet was resuspended in Buffer A solution (20 mM Tris (pH 7.9), 50 mM NaCl, 1% glycerol) containing 10 mM imidazole and lysed using a French press. After centrifugation of the lysate, the supernatant was applied to nickel-nitritrolactric acid-agarose (Qiagen) equilibrated with Buffer A and incubated at 4 °C overnight. The resin was washed with Buffer A solution containing 50 mM imidazole, and the polyhistidine-tagged Gcn2p-proteins were eluted with Buffer A solution containing 250 mM ethylene glycol. The low and high molecular weight gel filtration standards (Amersham Biosciences) and the Gcn2p-Ces were analyzed under identical conditions (0.4–0.5 ml/min, 200-μl sample load), and their relative mobilities were calculated as the ratio of (Ve/Vt) to (Ve/Vt), where Ve, Vt, and Vr are the elution, void, and total bed volumes, respectively. The molecular weight of the Gcn2p proteins was determined by comparison of their relative mobility to a straight line plot determined for the standards. Gcn2p-C was visualized by using SDS-polyacrylamide gels (14% polyacrylamide) and visualized by staining with Coomassie R-250. Gcn2p-C was also visualized by immunoblot analysis using polyclonal antibody that specifically recognizes the polyhistidine tag (Santa Cruz Biotechnology), horseradish peroxidase-labeled secondary antibody, and chemiluminescent substrate.

Expression and Purification of Gcn2-C Proteins—The plasmid p540 derivatives encoding wild type or mutant versions of Gcn2p-C were transformed into E. coli strain C43 (DE3) (pET28C). The plasmids used were introduced into yeast strain KY102 (MATa ura3-52 leu2 trp1-163). The purified Gcn2p solutions were buffered with 50 mM Tris-HCl (pH 7.9) 50 mM NaCl, and 1% (v/v) ethylene glycol. The low and high molecular weight gel filtration standards (Amersham Biosciences) and the Gcn2p-Ces were analyzed under identical conditions (0.4–0.5 ml/min, 200-μl sample load), and their relative mobilities were calculated as the ratio of (Ve/Vt) to (Ve/Vt), where Ve, Vt, and Vr are the elution, void, and total bed volumes, respectively. The molecular weight of the Gcn2-C proteins was determined by comparison of their relative mobility to a straight line plot determined for the standards.
Dimerization and Activation of Gcn2

Gcn2 Complementation in Yeast—To measure Gcn2p function, RY139 cells each expressing different Gcn2 alleles were grown to saturation in synthetic medium containing 2% glucose (SD) supplemented with the required amino acids (32). Cultures were diluted to an A600 of 0.25, and 7 μl of the samples were spotted onto agar plates containing SD medium supplemented with all 20 amino acids (SC), medium supplemented with 30 mM 3-aminitriazole, and all amino acids except histidine (SC + 3-AT) or SC medium containing 50 mM rapamycin (SC + Rap) or 1 mM NaCl (SC + NaCl). Alternatively, samples were spotted onto enriched medium YPD (32). Plates were incubated at 30°C, and growth was monitored over a period of 4–6 days.

Immunoblot Analysis—RY139-derived cells expressing the indicated Gcn2 allele were grown to mid-logarithmic phase in SD medium supplemented with leucine, isoleucine, valine, and tryptophan at 30°C. Cells were collected by centrifugation, washed with ice-cold water, and resuspended in a solution of 20 mM sodium phosphate (pH 7.2), 50 mM NaCl, 5 mM EDTA, 1 mM diethiothreitol, protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin), and phosphatase inhibitors (50 mM NaF and 40 mM β-glycerophosphate). Cells were lysed by using glass beads and vortexing for 2 min at 15-s intervals while maintaining the samples on ice. After a 2000 × g centrifugation to remove the glass beads and cell debris, the lysate was subjected to centrifugation at 16,000 × g for 30 min. The protein content of the cell lysate was measured using the Bradford method and bovine serum albumin as a standard (33).

For each protein sample, separated by electrophoresis in an SDS-polyacrylamide gel (12% polyacrylamide) and transferred to nitrocellulose filters. Broad range polypeptide markers (Bio-Rad) were used to measure the molecular weight of proteins. Immunoblot analyses were carried out using a polyclonal antibody that specifically recognizes phosphorylated eIF2α at Ser-51 (Research Genetics or StressGen), horseradish peroxidase-conjugated anti-rabbit IgG, and chemiluminescent substrate. To measure total levels of eIF2α, the same nitrocellulose membrane was stripped using a solution of 70 mM Tris (pH 6.7), 100 mM β-mercaptoethanol, and 2% SDS, and reprobed using a rabbit polyclonal antibody against recombinant yeast eIF2α (9). Gcn2p levels were measured in strain RY139 containing p722 and its derivatives encoding wild type or mutant forms of Gcn2 and pYB41. The cells were harvested in mid-logarithmic phase as before, and cell pellets were washed in ice-cold water. Due to observed high levels of degradation of full-length Gcn2, the cell pellets were boiled for 10 min immediately after the washing step. Protease inhibitors were added to the cell preparation, and the cells were lysed by using glass beads and vortexing. Lysates were clarified by centrifugation, and 40 μg of protein samples were separated using SDS-PAGE (7% polyacrylamide). Separated proteins were transferred to nitrocellulose membrane, and Gcn2p was visualized using Gcn2p-specific antibody that was generously provided by Dr. Alan Hinnebusch (National Institutes of Health).

Gcn4-lacZ Enzyme Assay—RY139 strains expressing wild type or mutant versions of Gcn2p and Gcn4-LacZ were grown to mid-logarithmic phase and then shifted to SD medium containing 30 mM 3-AT, 1 mM NaCl, 200 mg/ml rapamycin, or no stress agent. Following incubation at 30°C, the non-stressed cells were harvested after 4 h, and stressed cells were collected after 6 h. Cells were collected by centrifugation, resuspended in 250 μl of breaking solution (100 mM Tris-3-SC (pH 8.0), 20% glycerol, 1 mM β-mercaptoethanol, and 100 μM phenylmethylsulfonyl fluoride), and broken using glass beads and vortexing. Cell lysates were collected and clarified by centrifugation at 15,000 × g. To measure the β-galactosidase enzyme activity, 20 μl of extract was added to 980 μl of Z-buffer (100 mM sodium phosphate (pH 7.5), 10 mM KCl, 2 mM MgSO4, 40 mM KCl, 0.03% NaN3, 0.001% phenylmethylsulfonyl fluoride, and 1% Triton X-100), and the reaction was initiated by the addition of a 200-μl solution of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml o-nitrophenyl β-D-galactopyranoside in Z-buffer). The reaction was terminated following 20 and 60 min of incubation at 28°C by adding 0.5 ml of 1 M Na2CO3, and the absorbance of the reaction mixture was measured at 420 nm. Enzyme activity is represented as the nanomoles of o-nitrophenol/μl/30 min in the reaction and is consistent with a dimer (Fig. 2, A and D). The carboxyl terminus of Gcn2p was suggested to interact with itself in a yeast two-hybrid analysis, and deletion of sequences extending to residue 1536 reduced this association (14). We therefore expressed and purified recombinant protein containing Gcn2p residues 1536–1659 using the T7 promoter system in E. coli. This recombinant protein, we refer to as Gcn2-Cp, was purified using an amino-terminal polyhistidine sequence and nickel chelation resin (Fig. 1). Purified Gcn2-Cp was analyzed using an S-200 gel filtration column and found to elute in a buffer solution containing 50 mM NaCl with a molecular weight of 44,000, consistent with a dimer (Fig. 2, A and D). The carboxyl terminus of Gcn2p has been implicated in multiple functions in the mechanisms activating this eIF2 kinase in response to amino acid starvation (Fig. 1). To discern the role of the carboxyl terminus of Gcn2p in oligomerization, we expressed a recombinant protein containing Gcn2p residues 1498–1659 using the T7 promoter system in E. coli. This recombinant protein, we refer to as Gcn2-Cp, was purified using an amino-terminal polyhistidine sequence and nickel chelation resin (Fig. 1). Purified Gcn2-Cp was analyzed using an S-200 gel filtration column and found to elute in a buffer solution containing 50 mM NaCl with a molecular weight of 44,000, consistent with a dimer (Fig. 2, A and D). The carboxyl terminus of Gcn2p was suggested to interact with itself in a yeast two-hybrid analysis, and deletion of sequences extending to residue 1536 reduced this association (14). We therefore expressed and purified recombinant protein containing Gcn2p residues 1536–1659 and found it to be a size of 18,000 daltons by gel filtration, consistent with a monomer (Fig. 2E). We next analyzed Gcn2-Cp, extending from residues 1498 to 1659 in the sizing column in the presence of 1 mM NaCl, and we found that it continued to elute as a dimer (Fig. 2B). Similarly, 1 mM KCl was not effective in disrupting the dimer formed by Gcn2-Cp. However, in the presence of 0.1% SDS the Gcn2-Cp eluted as a monomer (data not shown). These results suggest that the Gcn2-Cp dimer interface is not ionic in nature but rather involves hydrophobic interactions. Consistent with this premise, the inclusion of the organic solvent ethylene glycol in the gel filtration buffer solution rendered Gcn2-Cp a monomer, suggesting the involvement of hydrophobic interactions (Fig. 2C).
fig. 2. hydrophobic residues at the carboxyl terminus contribute to the dimer interface of Gcn2p. Straight line plots (Kd versus log(MW)) were generated using the gel filtration standards (filled squares) in a 20 mM Tris-HCl (pH 7.9) buffered solution containing 50 mM NaCl (A), or 1 M NaCl (B), or 50 mM ethylene glycol (C). Open circles in A indicate the elution positions of wild type Gcn2-Cp (C-Wt) and mutant F1623D/A1624D (C-Mut.), representing a dimer and monomer, respectively. The elution position of Gcn2-Cp (C-Wt) on C indicates a monomer in ethylene glycol, and Gcn2-H + Cp (H + C-Wt) represents a dimer in high salt. D–K represent SDS-PAGE analysis of gel filtration fractions of the indicated proteins in a solution containing 50 mM NaCl. Equal volumes of the starting material (S) or the indicated S-200 fractions (fractions 26–36) were separated by SDS-PAGE, and the proteins were visualized by staining with Coomassie R-250 or immunoblot analysis using anti-histidine tag antibody. Arrows indicate the elution positions of the dimer and monomer forms of the protein.

These results suggest that residues 1498–1536 of Gcn2p contribute to oligomerization of the carboxyl terminus and suggest a role for hydrophobic interactions at the dimer interface.

Chemical cross-linking experiments using glutaraldehyde as a bi-functional cross-linker also showed that the Gcn2-Cp forms a dimer, and to a lesser extent higher order oligomers (Fig. 3A). With increasing concentrations of protein, the cross-linked Gcn2-Cp was visible by silver staining following SDS-PAGE. A reduction in the Gcn2-Cp monomer band and a concomitant appearance of the dimer band were also evident with increasing concentrations of glutaraldehyde (Fig. 3B). Cross-linking reactions carried out in the presence of 1 mM NaCl also gave a dimer form of the Gcn2-Cp, consistent with the gel filtration results (Fig. 3B). Furthermore, to discount the possibility that a polyhistidine tag was involved in the dimer formation, we removed the polyhistidine sequences from Gcn2-Cp by using a thrombin cleavage site engineered between the amino-terminal tag and Gcn2-Cp, and we found it equally competent to form dimers as judged by gel filtration and cross-linking (data not shown). As a control we found that purified polyhistidine-tagged eIF2α did not form oligomers in the cross-linking reaction under the same conditions. Finally, we note that the cross-linked Gcn2-Cp dimer co-eluted in the gel filtration at the same position as the purified recombinant protein, supporting the idea that the identified Gcn2-Cp dimer is the same oligomer complex between the gel filtration and the cross-linking protein visualized in the SDS-PAGE assay.

Hydrophobic Residues at the Carboxyl Terminus Contribute to the Dimer Interface of Gcn2p—The stability of the Gcn2-Cp dimer, even in the presence of high salt concentrations, suggested a role for hydrophobic interactions in the dimer interface. A hydrophathy plot was generated for Gcn2-Cp, revealing four regions of hydrophobicity, designated A–D, which were flanked by hydrophilic sequences (Fig. 4). The residues corresponding to the hydrophobic peaks were substituted with charged residues Asp or Glu. Residues in the predicted hydrophilic segments were changed to Ala. Secondary structure predictions and our prior circular dichroism analysis of the carboxyl-terminal portion of Gcn2p indicated extensive α-helical structures (19). Of four predicted α-helical regions, designated 1–4, two are included in portions of the hydrophobic regions (α-helices 2 and 3 in Fig. 4). The α-helices designated 1 and 4 form predicted amphipathic α-helices, and hydrophobic residues in the predicted amphipathic helices were substituted to Asp or Glu. Together, 16 mutant versions of Gcn2-Cp, each

![Fig. 3. Gcn2-Cp forms cross-linked dimers that are resistant to high salt concentrations.](image-url)
containing one or two residue substitutions, were expressed in E. coli and purified using nickel chelation resin. Mutant versions of Gcn2-Cp were analyzed by gel filtration, with representative elution profiles for four mutant proteins illustrated in Fig. 2, F–I, and the ability to dimerize was compiled in Fig. 4.

Residues in the hydrophobic segment B of Gcn2-Cp that were characterized in this mutant analysis include substitution of residues Y to D at positions 1542 and 1543 (I1542D/Y1543D), and residues VP to D at 1544 and 1545 (V1544D/P1545D). Both of these mutant versions of Gcn2-Cp blocked dimerization, suggesting that this segment participates in the dimer interface (Fig. 4). By contrast, Gcn2-Cp containing residue substitutions V1531D/V1532D in hydrophobic segment A was a dimer in gel filtration (Fig. 4), indicating hydrophobic segments in the carboxyl terminus contribute differentially to Gcn2 oligomerization. Residue substitutions in hydrophobic segments C and D represented by I1583D/T1584D, I1596D/S1597D, and I1598D/T1599D, each disrupted dimerization (Fig. 4). These results suggest that hydrophobic residues in three distinct regions of Gcn2-Cp are important contributors to oligomerization.

Four mutations were made in the predicted amphipathic α-helix 4 included F1623D/A1624D, A1624D/T1625E, I1627D/Y1628D, and L1631E/S1632D (Fig. 4). With the exception of Gcn2-Cp, with residue substitutions A1624D/T1625E, each mutation in this α-helical region disrupted dimerization (Fig. 2, F and I and Fig. 4). These results support the idea that the hydrophobic face of helix 4 contributes to the Gcn2 dimer interface. The three Gcn2-C mutations in the predicted amphipathic helix 1 (Fig. 2F and Fig. 4) were dimers, demonstrating that this portion of Gcn2p is dispensable for oligomerization.

By using co-immunoprecipitation assays, it has been reported (12) that there are regions within the HisRS-related domain that facilitate dimerization between this portion of Gcn2p. We therefore expressed and purified recombinant Gcn2p containing both the HisRS and carboxyl-terminal domains. This Gcn2-H+ Cp was a dimer as judged by gel filtration (Fig. 2B). Introduction of the residue substitutions I1542D/Y1543D located in the hydrophobic segment B of the Gcn2-H+Cp blocked dimerization as described for the Gcn2-Cp (Fig. 2J). By contrast dimerization was retained when the m2 mutation was introduced into the Gcn2-H+Cp (Fig. 2K). We conclude that the carboxyl terminus is a primary determinant for dimerization of Gcn2p.

**Dimerization Is Not Required for Gcn2p Association with Ribosomes—**Gcn2p was reported to associate with free 40 S and 60 S ribosomal subunits, 80 S particles, and polysomes as judged by analysis of cell lysates separated by sucrose gradient centrifugation (18, 19) (Fig. 5). This ribosomal association is facilitated by the carboxyl terminus of Gcn2p and is required for activation of Gcn2p eIF2 kinase in response to amino acid depletion. We wished to address whether Gcn2p carboxyl-terminal dimerization is a prerequisite for ribosomal association. Gcn2p dimerization mutants I1542D/Y1543D, I1596D/S1597D, and L1631E/S1632D displayed a distribution pattern in the sucrose gradient that was similar to that observed for wild type Gcn2p. By comparison, the dimer mutant F1623D/A1624D migrated in the top portion of the gradient and with the 40 S ribosome fraction. This pattern is comparable with that described for gcn2-605p (K1552L/K1553I/K1556I) or a version of Gcn2p deleted for these three lysine residues that impede interaction with ribosomes (18, 19). The Gcn2p mutant A1624D/T1625E also had a major peak at the 40 S ribosome fraction, and a second peak centered between the 60 S and 80 S regions. A final dimer-defective Gcn2p examined was I1583D/T1584D that displayed two peaks of distribution in the sucrose gradient that was similar to that observed for wild type Gcn2p. A predominant peak was observed in the region of the 80 S particle and the smaller portion of polysomes and a second peak centered about the 40 S ribosomal subunit. Together,
these results suggest that carboxyl-terminal dimerization is not obligate for Gcn2p association with ribosomes and that there are certain residue substitutions, such as F1623D/ A1624D, that impact multiple functions in the carboxyl terminus of Gcn2p.

Gcn2p Dimerization Is Required for eIF2α Phosphorylation in Response to Amino Acid Starvation—We next addressed whether carboxyl-terminal dimerization of Gcn2p is required for eIF2 kinase activity and induction of GCN4 translation. The entire panel of mutations was introduced individually into plasmid-encoded full-length Gcn2p and transformed into yeast strain RY139 (gcn2::LEU2). Wild type Gcn2p confers resistance to 3-AT, an inhibitor of histidine biosynthesis (Fig. 6). Previously, we showed that residue substitutions in Gcn2p that inactivate the kinase catalytic domain (K628R), the partial kinase region (Δ429–504), HisRS-related domain (gcn2-m2), or ribosome binding (gcn2-g605) render RY139-derived cells growth-sensitive to 3-AT (9, 10, 19, 24). Gcn2p mutants that disrupt dimerization, including those with residue alterations in the hydrophobic segments B–D, or in helix 4, were also unable to support growth under the amino acid deprivation conditions (Figs. 4 and 6). Gcn2p levels as judged by immunoblot were similar between wild type Gcn2p and these carboxyl-terminal mutants, indicating that loss of in vivo function was not due to lowered levels of these mutant proteins (Fig. 7). The Gcn2p residue changes in the hydrophilic segments that allowed dimerization supported growth on 3-AT. The sole exception was N1617A/S1618A, which can be attributed to a lack of Gcn2p under steady-state conditions (Fig. 7). Gcn2p is the only eIF2 kinase present in yeast, and therefore the level of phosphorylated eIF2α under stress conditions is a direct measure of its in vivo activity. To assess directly the kinase activity of Gcn2p, we measured phosphorylation of eIF2α in vivo using antibody specific to the phosphorylated form of this translation factor. RY139 cells expressing the panel of different Gcn2 alleles were grown under non-starvation conditions or subjected to 3-AT treatment. Cells expressing wild type Gcn2p showed increased eIF2α phosphorylation in response to this amino acid limiting condition, whereas the strain containing the kinase-inactive K628R had no eIF2 kinase activity in vivo (Fig. 8A). Levels of total eIF2α were unchanged in the non-starved or amino acid-deprived conditions (Fig. 8A). These results show that Gcn2p carboxyl-terminal mutants that facilitate growth on 3-AT medium retained the ability to phosphorylate eIF2α, whereas mutants that rendered RY139 sensitive to 3-AT were blocked for eIF2α phosphorylation.

Stimulation of GCN4 Expression in Response to Nutritional Stress Requires Gcn2p Carboxyl-terminal Dimerization—Gcn2p phosphorylation of eIF2α in response to histidine starvation induces GCN4 translation through a mechanism involving four short ORFs located in the 5’-noncoding region of the GCN4 mRNA. To measure this GCN4 translation, we utilized a GCN4-lacZ fusion containing all four upstream ORFs in RY139-derived cells expressing wild type or mutant forms of Gcn2 (Tables I and II). In response to histidine starvation, Gcn4-LacZ enzyme activity in wild type Gcn2 cells showed over an 8-fold increase in enzyme activity that was substantially diminished in cells deleted for Gcn2 (Table I). We also analyzed expression of the GCN4-lacZ fusion in which there were nucleotide substitutions in the initiation codons of each of the four upstream ORFs. These mutations render the upstream ORFs nonfunctional for translation control and any increase of Gcn4-LacZ enzyme activity would be attributable to transcriptional control (2, 30). In the absence of upstream ORFs, Gcn4-
Dimerization and Activation of Gcn2

We addressed whether Gcn2p dimerization is essential for eIF2α phosphorylation and activation of GCN4 expression in response to treatment with rapamycin or 1 M NaCl. Cells expressing each of the dimer-disrupted mutants showed low levels of GCN4-LacZ enzyme activity, whereas strains containing Gcn2p mutants that retained the ability to dimerize induced GCN4 expression in response to the rapamycin or sodium stress (Table II). The sole exception was A1624D/T1625E that showed about a 7-fold induction of GCN4-LacZ enzyme activity in response to amino acid limitation. In response to rapamycin, there was a partial diminishment of GCN4 translation, with a 4.5-fold increase and only a 2-fold increase in the presence of elevated sodium levels.

Exposure of wild type cells to rapamycin or 1 M NaCl induced phosphorylation of eIF2α to levels exceeding that measured for amino acid starvation (Fig. 8B). Minimal eIF2α phosphorylation was observed in the isogenic strain expressing the kinase-deficient gcn2-K628R protein, the m2 mutant Y1199L/R1120L, or the dimerization mutant F1623D/A1624D in response to each of the three stress conditions. Gcn2p mutant W1514D that retained the ability to oligomerize efficiently phosphorylated eIF2α upon exposure to rapamycin or elevated sodium levels. Most interesting, cells expressing the dimerization-proficient A1624D/T1625E induced eIF2α phosphorylation in response to 3-AT or rapamycin treatment, but not in response to exposure to 1 M NaCl, consistent with the differential expression of Gcn4-LacZ enzyme activity that was noted above (Fig. 8B). We conclude that Gcn2p eIF2α kinase activity and dimerization are required for activation of Gcn2p and GCN4 translation in response to a variety of different stress conditions.

Laboratory Strains Vary in Their Sensitivity to Rapamycin or Elevated Sodium Concentrations—We surveyed a number of different S. cerevisiae laboratory strains for growth sensitivity to rapamycin and elevated sodium levels. Consistent with previous reports, we found a range of growth rates in the YPD medium containing rapamycin. Strains F113 and BY4741 showed robust growth in the presence of rapamycin, and JC482 and EG328-1A, the parent strain of RY139, displayed sensitivity to this immuno-suppressant drug. Strains that were sensitive to rapamycin were also growth-impaired in the presence of high concentrations of sodium (Fig. 9A). The sole exception was strain H1515 that showed robust growth in the presence of NaCl but sensitivity to rapamycin. Each of these strains uniformly supported growth in the presence of elevated levels of an alternative salt, KCl, suggesting that this growth sensitivity was not a consequence of osmotic stress (Fig. 9B and data not shown). In the case of EG328-1A, a prototroph variant of this strain encoding wild type URA3, LEU2, and TRP1 still showed reduced growth when exposed to rapamycin or elevated levels of NaCl. This suggests that the growth sensitivity is not simply due to amino acid deficiency. Further supporting this idea is the observation that Gcn4-LacZ expression is significantly increased in response to rapamycin or elevated levels of NaCl.

stress conditions induce GCN4 expression via a mechanism requiring the upstream ORFs in the leader of the GCN4 mRNA and Gcn2p (Table I). Activation of Gcn2p during these two stress conditions are suggested to involve uncharged tRNA as RY139-derived strains expressing either gcn2-K628R or gcn2-m2 proteins were reduced for GCN4 translation compared with wild type Gcn2 cells (Table II). Additionally, deletion of the partial kinase region of Gcn2p lowers Gcn4-LacZ enzyme activity in response to exposure to rapamycin or elevated levels of sodium. Cherkasova and Hinnebusch (35) also reported that rapamycin regulates phosphorylation of Gcn2p by inhibition of the Tor pathway, suggesting additional mechanisms for regulating Gcn2p activation.

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Laboratory Strains Vary in Their Sensitivity to Rapamycin or Elevated Sodium Concentrations—We surveyed a number of different S. cerevisiae laboratory strains for growth sensitivity to rapamycin and elevated sodium levels. Consistent with previous reports, we found a range of growth rates in the YPD medium containing rapamycin. Strains F113 and BY4741 showed robust growth in the presence of rapamycin, and JC482 and EG328-1A, the parent strain of RY139, displayed sensitivity to this immuno-suppressant drug. Strains that were sensitive to rapamycin were also growth-impaired in the presence of high concentrations of sodium (Fig. 9A). The sole exception was strain H1515 that showed robust growth in the presence of NaCl but sensitivity to rapamycin. Each of these strains uniformly supported growth in the presence of elevated levels of an alternative salt, KCl, suggesting that this growth sensitivity was not a consequence of osmotic stress (Fig. 9B and data not shown). In the case of EG328-1A, a prototroph variant of this strain encoding wild type URA3, LEU2, and TRP1 still showed reduced growth when exposed to rapamycin or elevated levels of NaCl. This suggests that the growth sensitivity is not simply due to amino acid deficiency. Further supporting this idea is the observation that Gcn4-LacZ expression is significantly increased in response to rapamycin or elevated levels of NaCl.
even when all 20 amino acids are added to the growth medium or in the protoplastic strain (Fig. 10, A and B).

Consistent with an earlier report (36), deletion of GCN2 in fact rendered RY139 more resistant to sodium toxicity (Fig. 9). Similarly, loss of GCN2 function provides a growth advantage in response to rapamycin exposure. Growth resistance to rapamycin and sodium stress were also found in strains expressing a mutant form of eIF2α with Ala substituted for the phosphorylation site Ser-51 (S51A), and in cells deleted for Gcn3p, a non-essential subunit of eIF2B that facilitates association and inhibition of this guanine nucleotide exchange factor by phosphorylated eIF2α (Fig. 9C). We next sought to determine whether growth resistance of Gcn2p eIF2 kinase activity was due to induced GCN4 expression or, alternatively, due to blocked translation initiation that can accompany hyperphosphorylation of eIF2α. Strain RY290-3 deleted for GCN4 also revealed a growth resistance to either rapamycin or elevated sodium levels (Fig. 9B). Similar growth resistance phenotypes were found by using prototrophic (LEU2 TRP1 URA3) versions of the GCN4 or GCN2-deficient strains (data not shown). By comparison, related strains deleted for APG1, required for autophagy (37), or Pho85 protein kinase that induces nutrient stress responses (38, showed growth sensitivity to these two stress conditions (Fig. 9, B and C). These results suggest that at least in the EG328-1A strain background that enhanced GCN4 expression is an important contributor to growth sensitivity to rapamycin and 1 M NaCl. A final note is that Valenzuela et al. (34) originally observed that loss of GCN4 elicited growth resistance to rapamycin only in combination with a GLN3 deletion. To ascertain whether there is a previously unknown GLN3 mutation in our EG328-1A-derived strains, we assayed growth in the presence of the glutamine synthetase inhibitor L-methionine sulfoximine. Although known GLN3-deficient strains are growth-sensitive to L-methionine sulfoximine (39), we found proficient growth of the EG328-1A strain and its derivatives in the presence of this inhibitor, suggesting that there is functional GLN3 (data not shown).

Starvation for amino acids activates Gcn2p by a mechanism requiring the Gcn2p kinase and HisRS-related domains as well as the Gcn1p-Gcn20p complex that is proposed to be important for facilitating delivery of uncharged tRNA to Gcn2p. We next addressed whether these regulatory features are also important for activation of Gcn2p in response to treatment with rapamycin or elevated levels of NaCl. Cells expressing gcn2-K628R or gcn2-m2, defective for protein kinase and HisRS-related functions, respectively, were growth-resistant to rapamycin or 1 M NaCl (Fig. 6). By comparison both Gcn2p mutant strains were unable to grow during amino acid starvation con-
ditions induced by the addition of 3-aminotriazole. This pattern of growth resistance to rapamycin and sodium was also observed in cells deleted for either GCN1 or GCN20 (Fig. 9C).

We next assayed each of the carboxyl-terminal Gcn2p mutants for their growth on medium containing rapamycin or high sodium levels. Each of the dimerization-defective mutants, as represented by V1544D/P1545D, also supported growth in the presence of rapamycin or elevated NaCl levels (Figs. 4 and 6). With one exception, Gcn2p mutants that retained dimerization were growth-sensitive to rapamycin or high sodium levels (Fig. 4, and see representative strains F1511D and W1514D in Fig. 6). The sole exception was the A1624D/T1625E that contributed to growth in the presence of 3-AT, rapamycin, or 1M NaCl. Together these results suggest that many of the mechanisms by which Gcn2p is activated during amino acid starvation are required for induction by rapamycin and sodium stress conditions. These central features include binding of uncharged tRNA to the HisRS-related domain, dimerization through the carboxyl terminus of Gcn2p, and Gcn2p interaction with the Gcn1p-Gcn20p complex. Most interesting, there may be some features in the carboxyl terminus of Gcn2p that differentially impact growth between these different stress conditions.

Reduced K+/H11001 Levels Contribute to Increased GCN4 Translation—Although treatment of yeast cells with 1 M NaCl significantly induced GCN4 expression, exposure to the same concentration of 1 M KCl had no effect on GCN4 translation (Fig. 10C). This observation suggests that osmotic stress alone is not the basis for activation of Gcn2p activity. It has been reported that yeast cultured in the presence of 1M NaCl increase their intracellular concentrations of sodium from negligible levels to 150 mM within 1 h (40). Concomitant with this elevation in sodium levels, there is over a 2-fold reduction in the intracellular concentrations of K+/H11001. We were interested in determining whether changes in intracellular K+ concentrations, and its possible impact on the membrane potential and transporters, could increase GCN4 translation. To address this idea, we replaced the 7 mM KH2PO4 present in synthetic dextrose minimal medium with the same concentration of NaH2PO4, and we...
cultured the EG328-1A-derived cells for 6 h. We found an 8-fold increase in Gcn4-LacZ activity in cells grown in the minimal medium devoid of KCl compared with the KCl-supplemented medium (Fig. 10B). We also combined this KCl-deficient medium with 1 M NaCl and found a further increase in GCN4 expression, with a 10-fold increase over the non-stressed conditions. By adding increasing concentrations of KCl to this KCl-deficient medium containing 1 M NaCl, there was a progressive reduction in the translational expression of GCN4. We found with the addition of 50 mM KCl there was less than a 2-fold increase in Gcn4-LacZ expression compared with the non-stressed growth medium. These results suggest that depletion of intracellular levels of KCl can contribute to activation of Gcn2p and induced GCN4 translation.

**DISCUSSION**

Activation of Gcn2p in response to stress is proposed to involve a conformational transition involving contacts between the multiple domains of this eIF2 kinase. In this report we show that the carboxyl terminus of Gcn2p between residues 1498 and 1659 facilitates the stable oligomerization between Gcn2 polypeptides, and that this interaction is required for induced eIF2alpha phosphorylation and GCN4 translational expression in response to many different stress conditions (Figs. 2 and 4 and Table II). Gcn2p dimerization is resistant to high levels of salt and is facilitated by three distinct hydrophobic segments, designated B-D, and a proposed amphipathic helix. In addition to dimerization, activation of Gcn2p requires the carboxyl-terminal association with ribosomes (18, 19). However, the structural requirements for carboxyl-terminal dimerization and ribosome association appear to be at least in part distinct, as many Gcn2p mutations that blocked dimerization retained association with the translational machinery. Therefore, the carboxyl-terminal domain is multifunctional and integral to activation of Gcn2p eIF2 kinase via uncharged tRNA binding to the HisRS-regulatory domain.

**Dimerization Is a Conserved Feature in Activation of eIF2 Kinase Family Members**—Qiu et al. (12, 14) suggested that the carboxyl terminus of Gcn2p could interact with itself, the HisRS-related domain, and the protein kinase region. By using the carboxyl terminus of Gcn2p in yeast two-hybrid assays, it was reported that deletions between residues 1518–1537 and 1578–1597 were deleterious to Gcn2p self-interaction (14). Our mutational analysis is in agreement with these regions participating in Gcn2p dimerization. Hydrophobic segment B closely flanks the 1518–1537 deletion, and hydrophobic regions C and D are embedded within the 1578–1597 deletion (Fig. 4).

Dimerization is also a key step in activation of other members of the eIF2 kinase family. In the example of the mammalian eIF2 kinase PKR, double-stranded RNA produced during viral infection binds to two regulatory domains located amino-terminal to the protein kinase domain (1, 41, 42). The bound double-stranded RNA is proposed to form a bridge between two PKR polypeptides, facilitating trans-autophosphorylation at multiple sites, including Thr-446 and Thr-451 in the kinase activation loop of PKR (16, 43, 44). Such phosphorylation of PKR may contribute to its activated conformation leading to phosphorylation of eIF2alpha and lowered translation that prevents viral gene expression and viral replication. It is interesting to note that in addition to dimerization, the double-stranded RNA binding domains of PKR facilitate ribosome association, and this interaction with the translational machinery is proposed to increase the localized concentration of PKR that would enhance interactions between PKR polypeptides (45). Dimerization is also proposed to be important for activation of the eIF2 kinase PEK in response to endoplasmic reticulum stress (46, 47). In this case, release of inhibitory association of the endoplasmic reticulum chaperone BiP/GRP78 from the PEK regulatory domain facilitates oligomerization and trans-autophosphorylation of activation loop residues in the PEK catalytic domain.

Dimerization is part of the multistep mechanism inducing Gcn2p phosphorylation of eIF2alpha. Elevated levels of uncharged
tRNA that accumulate during amino acid limitation bind to the HisRS-related domain, invoking a proposed activated conformational change in the Gcn2p kinase domain. It is suggested that the activated Gcn2p conformation is accompanied by trans-autophosphorylation of activation loop residues, Thr-882 and Thr-887, analogous to those described for PKR (16, 43). However, our studies suggest that the very stable Gcn2p dimerization is mediated through protein-protein interactions and does not require ligands as noted for PKR. The parallels between the multifunctional regulatory domains in Gcn2p and PKR suggest that there are common steps in their mechanisms of activation. Supporting this model we found that the carboxyl terminus of Gcn2p can functionally replace the regulatory domain of PKR. Expression of this Gcn2-Cp-PKR chimera in yeast devoid of endogenous GCN2 led to eIF2α hyperphosphorylation and reduced translation initiation. Introduction of Gcn2p dimerization mutations into the Gcn2-Cp-PKR fusion inhibited phosphorylation of eIF2α and relieved the growth inhibition.

Stress Signals Activating Gcn2p eIF2α Kinase Activity—Activation of Gcn2p occurs in response to environmental stresses that appear to have little in common. In the case of amino acid starvation, the resulting elevated levels of uncharged tRNA associate with the HisRS-related domain, leading to a proposed enhancement of kinase substrate interaction and phosphorylation of eIF2α. The fact that functions associated with the HisRS-related, partial kinase, and carboxyl-terminal domains are also required for Gcn2p phosphorylation of eIF2α in response to rapamycin and elevated levels of NaCl suggests that there are common features in each of these stress activation mechanisms. It is noteworthy that with the one exception each of the carboxyl-terminal mutations that blocked induction of GCN4 translation in response to amino acid limitation also impeded GCN4 expression in response to exposure to rapamycin and 1 M NaCl (Table II). The sole exception was the A1624D/T1625E mutation that catalyzed eIF2α phosphorylation in cells treated with 3-AT or rapamycin but not in response to 1 M NaCl (Fig. 8B). Furthermore, although there was a strong correlation between induced eIF2α phosphorylation and GCN4 expression and growth sensitivity to rapamycin or 1 M NaCl, the Gcn2p A1624D/T1625E cells were growth-resistant to 3-AT and rapamycin and to a lesser extent to 1 M NaCl (Fig. 6). Together, the A1624D/T1625E mutations support the idea that there are some regulatory differences in Gcn2p activation in response to 1 M NaCl compared with rapamycin and nutrient stresses.

Common stress signals activating Gcn2p in response to amino acid starvation and exposure to rapamycin or 1 M NaCl are not well understood. Synthesis or uptake of amino acids may be impaired during treatment with rapamycin or elevated NaCl levels, increasing the concentration of uncharged tRNA in the cell. Rapamycin induces the degradation of the tryptophan permease, Tat2p, through activation of the Npr1 protein kinase (48). Similarly, elevated levels of NaCl reduce uptake of many different amino acids (36, 49). Such reduced import of amino acids could lower the levels of free amino acids and charged tRNAs in yeast. Arguing against this amino acid limitation model is the observation that GCN4 expression was induced in prototrophic strains in response to either rapamycin or 1 M NaCl (Fig. 10B). Rapamycin and Na+ stress-induced GCN4-lacZ expression also occurred when the medium was supplemented with excess levels of all 20 amino acids (Fig. 10A). Furthermore, it has been reported that high concentrations of Na+ or K+ equally reduce amino acid uptake (36); however, only Na+ treatment enhances Gcn2p activity and GCN4 expression (Fig. 10C) (36). These results support the idea that perturbation of cellular processes other than those directly affecting amino acid synthesis and import contribute to activation of Gcn2p during rapamycin and NaCl-induced stress.

Cherkasova and Hinnebusch (35) reported that rapamycin induces Gcn2p activity in non-starved cells by blocking Tor-mediated phosphorylation of Gcn2p at Ser-577. This Gcn2p phosphorylation is not thought to be mediated directly by Tor proteins but rather through an unknown protein kinase that is downstream of Tor and Tap42p-regulated type 2A and type 2A-related protein phosphatases. Strongly supporting the idea that phosphorylation of Gcn2p contributes to its activation is the report that an alanine substitution at Ser-577 leads to induced eIF2α phosphorylation and GCN4 expression independent of rapamycin exposure and nutrient availability (35). It is important to note that nutritional stresses that activate Gcn2p, including amino acid or purine starvation, do not lead to dephosphorylation of Gcn2p at Ser-577 (35). Therefore, the precise nutritional conditions regulating Tor-directed control of GCN4 translation remain to be determined. Uncharged tRNA may also contribute to Gcn2p activation in response to rapamycin. Supporting this premise are the observations that yeast containing gcn2-m2 or deletions in GCN1 or GCN20, proposed to be required for delivery of uncharged tRNA to Gcn2p, are blocked for general control in response to rapamycin exposure (Table II and Fig. 9) (36). However, to date there are no reports that rapamycin reduces the efficiency of aminoacylation of tRNA (20).

There are several possible explanations for the activation of Gcn2p in yeast treated with elevated NaCl concentrations. Osmotic stress alone does not appear to be the primary rationale for Gcn2p activation as treatment with elevated concentrations of NaCl, but not KCl, enhances GCN4 translation (Figs. 9A and 10C). High intracellular Na+ concentrations have been reported to impede many enzymatic processes in vitro, including aminoacylation of tRNAs, and such inhibitory processes may contribute to the in vivo activation of Gcn2p (50–52). However, elimination of K+ from the medium can itself induce GCN4 expression, suggesting that Na+ toxicity alone is not sufficient for activation of Gcn2p (Fig. 10C). Furthermore, the addition of increasing amounts of KCl to the 1 M NaCl-supplemented medium significantly reduces GCN4 expression, indicating that the Na+/K+ ion balance plays a pivotal role in activation of Gcn2p. Reduced intracellular K+ concentrations accompanying incubation of yeast cells in 1 M NaCl is thought to be due to the influx of Na+ through pathways that function in the cellular uptake of K+ (53). Such transport changes would impact the electric potential across the plasma membrane that drives many cellular processes, including nutrient transport and compartmentalization within the cell.

Consequences of Induced GCN4 Expression in Response to Different Stress Conditions—Gcn4p has been referred to as the “master regulator” of a multilayered program of gene regulation designed to alleviate nutrient deprivation. The core layer of Gcn4p transcriptional control involves genes directly contributing to the synthesis of amino acids. However, a DNA microarray study by Narajanan and co-workers (3, 54) reported that of the 539 genes whose transcription requires Gcn4p for full induction in response to amino acid depletion only 73 contribute to amino acid biosynthesis. Therefore, the transcriptional control by Gcn4p exceeds beyond core amino acid synthetic genes. These additional genes are involved in intermediary metabolism related to amino acid and purine biosynthesis, autophagy important for reclamation of materials in the cytoplasm, permeases required for transport of amino acids,
and members of the mitochondrial carrier family involved in metabolite transport between this organelle and the cytoplasm. Gcn4p is also suggested to work in concert with the Tor pathway to activate genes required for catabolism of poor nitrogen sources, as well as repression of genes encoding ribosomal proteins and translation factors (3, 35). Thus, the linkage between Tor and Gcn2p provides a mechanism of cross-talk between different nutrient-sensing pathways. 

The central role of Gcn4p in the yeast strategy for recognizing and coping with nutritional deficiencies provides a clear rationale for why strains deficient for GCN4, or those genes required for induced expression of this transcription factor, are impaired for growth during amino acid deficiency (Fig. 3C). By contrast, it is problematic as to why deletions in these GCN4-activating genes result in growth resistance to rapamycin or elevated concentrations of NaCl. This uncertainty is compounded by the fact that laboratory strains that were derived at least in part from strain S288C display great differences in growth sensitivity to NaCl or Na⁺ toxicity (Fig. 9A). In our limited survey we found that those strains displaying growth sensitivity to 1 M NaCl were also sensitive to rapamycin, with the exception of strain H1515 that was selectively resistant to elevated Na⁺ levels (Fig. 9A).

The presumed genetic variance in laboratory strains has contributed to conflicting conclusions in the literature. For example, it was reported that while gcn2A or SUL2-S1A alleles increased rapamycin resistance in strains derived from the Research Genetics background, deletion of GCN4 had no effect (35). It was concluded that reduced protein synthesis accompanying hyperphosphorylation of eIF2α leads to the slow growth phenotype associated with growth of wild type GCN2 strains grown in the presence of rapamycin. By contrast in strains derived from EG328-1A, induced Gcn4p activity is a primary reason for growth sensitivity to rapamycin (Fig. 9B). The contribution of Gcn4p could be due to its regulation of genes that contribute to rapamycin sensitivity. This may be due to direct Gcn4p binding at the promoters of these genes, leading to activation or repression of transcription. Alternatively, this Gcn4p function could be indirect. Gcn4p is known to bind to multiple co-activators (55–57), and increased expression of Gcn4p in cells exposed to rapamycin could lead to sequestration of one or more of these transcription factors required for appropriate transcription of stress resistance genes. Such explanations could also be the basis for GCN4 activity leading to enhanced sensitivity to 1 M NaCl.

There are also differences in the literature regarding the contribution of general control genes to growth resistance to Na⁺ toxicity. Goosens et al. (36) reported that deletion of Gcn2 contributed to yeast resistance to elevated concentrations of NaCl in cells derived from strain W303-1A. A complication of this laboratory strain is that the wild type cells are quite resistant to NaCl. In order to increase the Na⁺ sensitivity of W303-1A-derived cells it was necessary to introduce a mutation in ENA1, encoding a Na⁺-pumping ATPase critical for salt tolerance. By contrast, Pascual-Ahuir et al. (58) found that deletion of GCN4 in the same W303-1A strain background rendered cells more sensitive to 1 M NaCl or 1 M KCl but not other osmotic stress conditions. The basis for the stress sensitivity appears to be that Gcn4p competes with the transcriptional repressor Sko1p for binding to a cAMP-response element in the HAL1 promoter (58). Hal1p functions in the maintenance of cellular Na⁺/K⁺ ion balance and confers salt tolerance when overexpressed in yeast cells. Loss of Gcn4p activity would prevent HAL1 expression in response to high salt concentrations. The apparent conflict between the Na⁺ toxicity phenotypes of Δgcn2 and Δgcn4 mutants in the W303-1A strain is not easily reconciled. It is possible that modest increases in GCN4 expression enhance tolerance to NaCl, whereas very high levels of Gcn4p and the accompanying translation inhibition resulting from Gcn2p hyperphosphorylation of eIF2α may impede growth.

It is also noteworthy that Gcn2p can contribute to the regulation of the HOG mitogen-activated protein (MAP) kinase pathway important for responding to osmotic stress in yeast. Phosphorylation of Hog1p during osmotic stress leads to its translocation to the nucleus where it enhances stress gene expression through phosphorylation of transcription factors such as Hot1p and Sko1p (59). The immuno-suppressant FK506 was found to enhance Gcn2p stimulation of GCN4 expression through tryptophan starvation of strains that are deficient for the biosynthesis of amino acids (59, 60). When this tryptophan deprivation is coupled with osmotic stress, there is a Gcn2p-mediated hyperphosphorylation of Hog1p that appears to interfere with the Hog1p-dependent gene expression. Conversely, Hog1p appears to be required for full phosphorylation of eIF2α in response to tryptophan limitation. This cross-talk between the general control and HOG mitogen-activated protein kinase pathways may be through direct Gcn2p phosphorylation of Hog1p, and vice versa, or alternatively through intermediary protein kinases or phosphatases. Together these studies suggest that Gcn2p can impact salt tolerance through regulatory mechanisms that may involve phosphorylation of proteins other than eIF2α.

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