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Genetics

Mutations in the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) that overcome the inhibitory effect of eIF-2α phosphorylation on translation initiation

(protein kinase GCN2/*GCN4*/guanine nucleotide exchange factor)

CARLOS R. VAZQUEZ DE ALDANA, THOMAS E. DEVER, AND ALAN G. HINNEBUSCH

Section on Molecular Genetics of Lower Eukaryotes, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Phosphorylation of eIF-2α in *Saccharomyces cerevisiae* by the protein kinase GCN2 leads to inhibition of general translation initiation and a specific increase in translation of *GCN4* mRNA. We isolated mutations in the eIF-2α structural gene that do not affect the growth rate of wild-type yeast but which suppress the toxic effects of eIF-2α hyperphosphorylation catalyzed by mutationally activated forms of GCN2. These eIF-2α mutations also impair translational derepression of *GCN4* in strains expressing wild-type GCN2 protein. All four mutations alter single amino acids within 40 residues of the phosphorylation site in eIF-2α; however, three alleles do not decrease the level of eIF-2α phosphorylation. We propose that these mutations alter the interaction between eIF-2 and its recycling factor eukaryotic translation initiation factor 2B (eIF-2B) in a way that diminishes the inhibitory effect of phosphorylated eIF-2 on the essential function of eIF-2B in translation initiation. These mutations may identify a region in eIF-2α that participates directly in a physical interaction with the GCN3 subunit of eIF-2B.**

In mammalian cells, phosphorylation of eIF-2α on Ser-51 leads to an inhibition of protein synthesis at the initiation step. The phosphorylated form of eIF-2 reduces the ability of a second initiation factor, eukaryotic translation initiation factor 2B (eIF-2B), to catalyze GDP–GTP exchange on eIF-2. Because only the GTP-bound form of eIF-2 can deliver initiator-tRNA^{Met} to the ribosome, translation initiation is impaired. This regulatory mechanism is used to reduce total cellular protein synthesis in response to both viral infection and certain kinds of stress, including heme deprivation, heat shock, and amino acid starvation (for review, see ref. 1).

In yeast, eIF-2α is phosphorylated on Ser-51 by the protein kinase GCN2 when cells are starved for an amino acid, and this stimulates the translation of *GCN4* mRNA (2). *GCN4* encodes a transcriptional activator of the amino acid biosynthetic genes for which expression is subject to general amino acid control (for review, see ref. 3). When amino acids are abundant, translation of *GCN4* mRNA is repressed by short upstream open reading frames (uORFs) present in its leader. It is thought that ribosomes translate the uORF closest to the 5′ end of the mRNA (uORF 1), reinitiate at one of the remaining three uORFs in the leader, and fail to reinitiate again further downstream at *GCN4*. Under starvation conditions, however, ribosomes ignore the start codons at uORFs 2–4 and reinitiate at *GCN4* instead (3, 4). It was proposed that the start codons at uORFs 2–4 are ignored because phosphorylation of eIF-2α by protein kinase GCN2 lowers the level of active eIF-2 in the cell and thereby increases the time required to bind initiator tRNA to ribosomes scanning downstream from uORF 1 (2).

We previously described mutations that increase the ability of kinase GCN2 to phosphorylate eIF-2α in the absence of amino acid starvation, causing constitutive derepression of *GCN4* translation (5, 6). The most potent of these *GCN2^c* mutations decrease the cellular growth rate and inhibit general translation initiation. The same phenotypes were observed when two different mammalian eIF-2α kinases were expressed at high levels in yeast cells (7, 8). In all cases, the toxic effects of eIF-2α hyperphosphorylation were completely reversed by substituting Ser-51 in eIF-2α with a nonphosphorylatable alanine residue (2, 6–8).

The GCN3 protein is required in addition to GCN2 kinase for increased translation of *GCN4* mRNA in amino acid-starved cells (3). Recent results indicate that GCN3 protein and four other translational regulators of *GCN4*, known as GCD1, GCD2, GCD6, and GCD7 proteins, are subunits of the yeast equivalent of mammalian eIF-2B (9–11). This complex is required *in vivo* for general translation initiation (9, 12) and, *in vitro*, catalyzes guanine nucleotide exchange on eIF-2 (11). In addition, GCD6 protein exhibits strong sequence similarity with the largest subunit of rabbit eIF-2B (10). Interestingly, inactivation of the GCN3 subunit of eIF-2B has no effect on cellular growth rate (3) or general translation initiation (13); however, this inactivation overcomes the high-level *GCN4* expression and slow-growth rate conferred by *GCN2^c* alleles (6, 14) or by expression of mammalian eIF-2α kinases in yeast (8). These findings led to the idea that GCN3 is a regulatory subunit of the complex that mediates the inhibitory effect of phosphorylated eIF-2 on eIF-2B catalytic activity (8). Although GCN3 is dispensable for eIF-2B function, mutant forms of the protein encoded by *gcn3^c* alleles confer reduced rates of general translation initiation (13).

In an effort to identify other factors involved in eIF-2α phosphorylation and its inhibitory effect on translation initiation, we have isolated additional mutations that suppress the slow-growth phenotype of a *GCN2^c* allele. The suppressor mutations we obtained map in five unlinked loci in addition to *GCN3* (C.R.V. and A.G.H., unpublished work). In this report, we describe four suppressor mutations that alter the structural gene for eIF-2α in yeast, known as *SUI2*. Surprisingly, three of these suppressors overcome the effects of activated eIF-2α kinases on translation initiation without reducing the level of eIF-2α phosphorylation. We propose that the phosphorylated forms of these mutant eIF-2α proteins lack the ability to inhibit eIF-2B catalytic function. The

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Abbreviations: eIF-2α, α subunit of eukaryotic translation initiation factor 2; uORF, upstream open reading frame; 3-AT, 3-aminotriazole; allele *XnY* indicates an allele with an *X* → *Y* substitution at position *n*; eIF-2B, eukaryotic translation initiation factor 2B; dsRNA-PK, double-stranded-RNA-activated eIF-2α kinase.

amino acids altered by these mutations may participate directly in the physical interaction between eIF-2 and eIF-2B that is central to the regulation of protein synthesis by eIF-2α kinases in eukaryotic cells.

MATERIALS AND METHODS

Spontaneous *SUI2* mutations that suppress the slow-growth phenotype of *GCN2^c-E532K,E1522K* (6) were isolated by selecting fast-growing revertants of two different strains harboring this *GCN2^c* allele (indicated for each *GCN2^c*, *gcn3^c*, and *SUI2* allele is the wild-type amino acid, its position relative to the N terminus, and the substituting allele, in that order). Revertants H1822 and H1825 were derived from strain H1691 (*MATa ino1 ura3-52 GCN2^c-E532K,E1522K* ⟨*HIS4–lacZ*⟩), and revertants H1823 and H1824 were obtained from strain H1627 (*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K* ⟨*HIS4–lacZ*⟩). Genetic characterization of these and other revertants will be described elsewhere. The *SUI2* suppressors were cloned by PCR using primers complementary to the 5′ (5′-GCCGAATTCAGTTC-TACTGGGATG-3′) and 3′ (5′-GCCCTCGAGGCCAAATG-TACAAGGTG-3′) flanking regions of *SUI2*. The 1.5-kb amplified fragments were cloned between the *Eco*RI and *Xho* I sites of plasmid pRS316 (15), and the complete protein-coding region was sequenced (16) by using oligonucleotide primers. Several independent plasmids derived from each PCR reaction were sequenced to confirm that the mutations were not generated by the PCR. The 201-bp *Sal* I–*Hin*dIII fragment of *SUI2* containing each of the mutations from H1822, H1823, and H1824 was introduced into wild-type *SUI2* on the single-copy-number *LEU2* vector pSB32 (17) to create plasmids p1349 (*SUI2-V89I*), p1350 (*SUI2-L84F*), and p1351 (*SUI2-R88C*). The 658-bp *Eco*RI–*Sal* I fragment cloned from H1825 was inserted in the same vector, creating p1352 (*SUI2-I58M*). Plasmids p1098 and p1105 carry the *SUI2-S51A* and *SUI2-S48A* alleles, respectively (2). Strain H1925 (*MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn2Δ*, p1108[*GCN4–lacZ TRP1*] integrated at *trp1-Δ63*, p919[*SUI2 URA3*]) was transformed (18) with the *LEU2* plasmids containing the *SUI2* suppressor alleles, and the transformants were transferred to medium containing 5-fluoroorotic acid to evict the *URA3* plasmid carrying wild-type *SUI2* (19). This procedure yielded a set of isogenic strains containing the *SUI2* suppressor alleles on the *LEU2* plasmids as the only copy of *SUI2*. Strains H1816 (wild-type *SUI2*) and H1817 (*SUI2-S51A*) (2) are also isogenic to H1925, as is H2116 (*MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn2Δ gcn3Δ* p1108[*GCN4–lacZ, TRP1*] integrated at *trp1-Δ63*, p919[*SUI2, URA3*]), obtained as a Ura$^+$ Leu$^-$ segregant of H2065 (8).

Plasmids carrying *GCN2^c* alleles on the low-copy-number *URA3* vector pRS316 containing the mutations *GCN2^c-E532K,E1522K* (plasmid p1056), *GCN2^c-R699W,D918G, E1537K* (plasmid p1053), and *GCN2^c-M719V,E1537G* (plasmid p1052) have been described (6).

RESULTS

Cloning and Sequence Analysis of *SUI2* Alleles That Suppress the Slow-Growth Phenotype of a *GCN2^c* Mutation. Four chromosomal mutations in the *SUI2* gene were identified that overcome the toxic effects of a constitutively activated form of the protein kinase encoded by *GCN2^c-E532K,E1522K*, which contains mutations in the protein kinase and C-terminal domains of GCN2 (6). These mutant alleles of *SUI2* were cloned by PCR and inserted into low-copy-number plasmids for further examination. DNA sequence analysis revealed that each of the four alleles contained a different missense mutation in the N-terminal one-third of the eIF-2α protein. The segment encompassing these mutations is highly conserved between human, rat, and yeast eIF-2α (Fig. 1). The Ile-58 residue substituted in *SUI2-I58M* is very close to the GCN2-dependent phosphorylation site at Ser-51; the other three mutations are clustered near a potential phosphorylation site for the cAMP-dependent protein kinase at Ser-90.

To demonstrate that the cloned *SUI2* alleles were sufficient to confer the suppressor phenotype, we introduced each plasmid-borne suppressor, the wild-type allele, or the *SUI2-S51A* allele (encoding alanine instead of serine at position 51) into a strain lacking chromosomal copies of both *SUI2* and *GCN2*. When a plasmid containing *GCN2^c-E532K,E1522K* was introduced into these strains, we found that all four suppressor *SUI2* alleles overcame the slow-growth phenotype caused by this kinase to the same extent seen previously for the *SUI2-S51A* allele (2) (Fig. 2*A*). We then analyzed the general amino acid control response in these transformants by scoring growth in the presence of an inhibitor of histidine biosynthesis, 3-aminotriazole (3-AT). Derepression of *GCN4* translation is required for resistance to 3-AT; thus, strains lacking *GCN2* or its phosphorylation site on eIF-2α, which cannot derepress *GCN4*, are sensitive to 3-AT. The *SUI2-L84F* allele conferred a 3-AT-sensitive phenotype in strains containing either wild-type *GCN2* or *GCN2^c-E532K,E1522K* (Fig. 2*B*), the same effect observed previously for the *SUI2-S51A* allele (2). The *SUI2-V89I* and *SUI2-I58M* transformants containing wild-type *GCN2* were not as sensitive as the *SUI2-L84F GCN2* and *SUI2-S51A GCN2* strains, and the *GCN2^c* strains containing the *SUI2-V89I* and *SUI2-I58M* alleles were resistant to 3-AT. Both the *GCN2* and *GCN2^c* strains bearing *SUI2-R88C* were 3-AT-resistant. Thus, the four *SUI2* suppressors impaired the general control response to different extents, with *SUI2-L84F* having the greatest effect and *SUI2-R88C* being the least impaired.

The *SUI2* Suppressor Mutations Impair Derepression of *GCN4* Expression. To determine the effects of the *SUI2* mutations on *GCN4* expression, we assayed a *GCN4–lacZ* fusion present in the strains described above containing either *GCN2* or *GCN2^c-E532K,E1522K* and each of the plasmid-borne *SUI2* alleles. As expected, the wild-type *SUI2* strain bearing wild-type *GCN2* showed ≈8-fold derepression of *GCN4–lacZ* expression when starved for histidine by 3-AT, whereas the corresponding *GCN2^c* strain was constitutively derepressed (Table 1). The *SUI2-S51A* mutation impaired derepression of *GCN4–lacZ* expression in both the *GCN2* and *GCN2^c* strains (2). The *SUI2-L84F* allele also

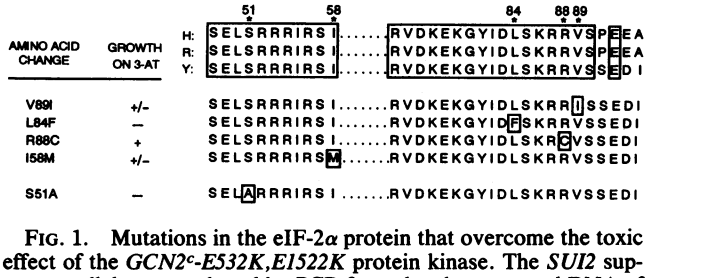

FIG. 1. Mutations in the eIF-2α protein that overcome the toxic effect of the *GCN2^c-E532K,E1522K* protein kinase. The *SUI2* suppressor alleles were cloned by PCR from the chromosomal DNA of four fast-growing revertants of *GCN2^c-E532K,E1522K SUI2* strains. The amino acid changes in eIF-2α caused by the *SUI2* suppressors are given in the first column. The second column compares the ability of the four revertants and a strain containing the *SUI2-S51A* allele (2) to grow on 3-AT medium, which is directly correlated with the level of *GCN4* expression. The parental strains containing *GCN2^c-E532K,E1522K* and wild-type *SUI2* gave a + level of growth on 3-AT medium. To the right of the columns is the amino acid sequence of eIF-2α (in single-letter code) from positions 48–94 in human (H; ref. 20), rat (R; ref. 20), and *Saccharomyces cerevisiae* (Y; ref. 21), with the conserved residues enclosed in boxes. Beneath this alignment, the amino acid sequence in this interval is shown for the *SUI2* suppressor alleles and for *SUI2-S51A*, with the altered amino acids also enclosed in boxes.

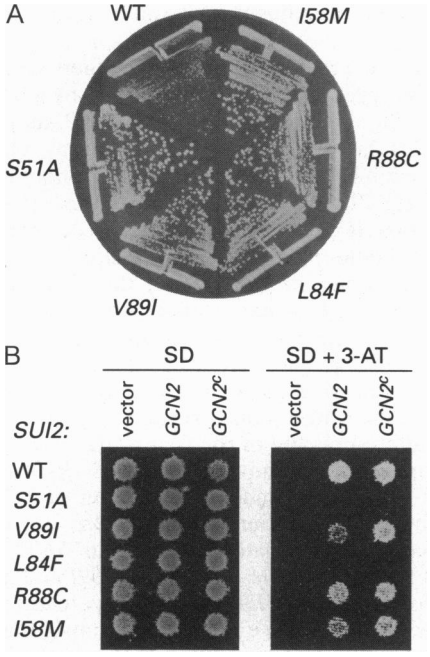

FIG. 2. Effects of eIF-2α suppressor mutations on cellular growth rate under nonstarvation conditions and resistance to 3-AT in strains containing *GCN2c-E532K,E1522K*. The indicated *SUI2* alleles were introduced on low-copy-number plasmids into strain H1925 that is deleted for the chromosomal copies of both *SUI2* and *GCN2*. The resulting strains H1926 (*SUI2-V89I*), H1927 (*SUI2-L84F*), H1928 (*SUI2-R88C*), and H1929 (*SUI2-I58M*) and the isogenic control strains H1816 (*SUI2*) and H1817 (*SUI2-S51A*) were transformed with plasmid p1056 containing the *GCN2c-E532K,E1522K* allele, with plasmid p585 containing *GCN2*, or with vector alone. (*A*) Strains carrying the *GCN2c-E532K,E1522K* allele and the indicated *SUI2* allele were streaked on SD plates and incubated for 2 days at 30°C. (*B*) Patches of transformants carrying YCp50 or the indicated *GCN2* and *SUI2* alleles were grown to confluence on SD plates and replica-plated to 3-AT plates (10 mM 3-AT) and SD plates and incubated for 3 days at 30°C. WT, wild type.

significantly reduced derepression of *GCN4*, whereas the *SUI2-V89I* and *SUI2-I58M* alleles had lesser effects, and the *SUI2-R88C* allele was the least effective at reducing *GCN4–lacZ* expression under derepressing conditions. These results are in complete accord with the 3-AT sensitivity observed for the *SUI2* suppressor strains, shown in Fig. 2*B*.

The *SUI2* Mutations Suppress the Slow-Growth Phenotype Conferred by Different *GCN2c* Alleles and a Human eIF-2α Kinase. To test whether the eIF-2α proteins encoded by the *SUI2* suppressors have a specific interaction with the protein kinase encoded by *GCN2c-E532K,E1522K*, we tested the ability of the *SUI2* mutations to suppress the slow-growth phenotype associated with other *GCN2c* alleles. The same strains described above containing plasmid-borne *SUI2* suppressors were transformed with plasmids carrying the *GCN2c-R699W,D918G,E1537K* or *GCN2c-M719V,E1537K* alleles. Both plasmids conferred a greater growth defect and higher levels of eIF-2α phosphorylation (6) in the wild-type *SUI2* strain than was seen for *GCN2c-E532K,E1522K*. All four *SUI2* suppressors completely overcame the slow-growth phenotype of *GCN2c-R699W, D918G,E1537K* and *GCN2c-M719V,E1537K*, but only the *SUI2–L84F* allele also conferred 3-AT sensitivity in the presence of these two highly activated *GCN2c* alleles (data not shown).

The *SUI2* suppressors can also overcome the slow-growth phenotype conferred on yeast by expression of the human double-stranded-RNA-activated eIF-2α kinase (dsRNA-PK; also known as DAI, dsI, and P68) under the control of a galactose-inducible promoter. As has been shown (8), a wild-type *SUI2* strain containing the dsRNA-PK construct grew very slowly on galactose medium, whereas one containing the *SUI2-S51A* mutation grew at the same rate as the parental strain containing an inactive dsRNA-PK construct with a Lys-296 → Arg substitution in the kinase domain (Fig. 3). We showed previously that the *SUI2-S51A* mutation prevents phosphorylation of yeast eIF-2α by dsRNA-PK, accounting for suppression of the slow-growth phenotype (8). The *SUI2-L84F* allele also completely suppressed the toxic effect of expressing dsRNA-PK in yeast cells (Fig. 3), whereas the other three *SUI2* alleles only partially suppressed this phenotype. The *SUI2-R88C* allele was the least effective suppressor of the human eIF-2α kinase (Fig. 3). The fact that the four *SUI2* mutations showed the same relative efficiencies of suppression for three different *GCN2c* alleles and for the human dsRNA-PK suggests that the mechanism of suppression does not involve allele-specific interactions

Table 1. *GCN4–lacZ* expression in *GCN2c* or *GCN2* strains containing *SUI2* suppressor alleles

		GCN4–lacZ expression, units			
		GCN2c-E532K,E1522K		*GCN2*	
Strain (allele)	Plasmid	R	DR	R	DR
H1816 (*SUI2*)	p1097	140	170	12	94
H1817 (*SUI2-S51A*)	p1098	7	17	8	20
H1926 (*SUI2-V89I*)	p1349	31	46	10	49
H1927 (*SUI2-L84F*)	p1350	19	32	12	30
H1928 (*SUI2-R88C*)	p1351	54	100	16	71
H1929 (*SUI2-I58M*)	p1352	30	58	12	45

Isogenic yeast strains H1816, H1817, H1926, H1927, H1928, and H1929 carrying the indicated *SUI2* alleles on low-copy-number plasmids were transformed with the single-copy plasmid p585 (5) containing wild-type *GCN2* or p1056 containing *GCN2c-E532K,E1522K* and grown for 8 hr under nonstarvation conditions (SD minimal medium) in which the general control system is repressed (R) or for 6 hr under conditions of histidine starvation (SD plus 10 mM 3-AT) in which the system is derepressed (DR). Expression of β-galactosidase from a *GCN4–lacZ* fusion integrated in the chromosome at *TRP1* was measured in cell extracts prepared from the different strains (22). Each value is the average obtained from two different transformants; the individual measurements varied from the mean values by 25% or less. Units of enzyme activity are given as nmol of *o*-nitrophenyl β-D-galactopyranoside cleaved per min per mg of protein.

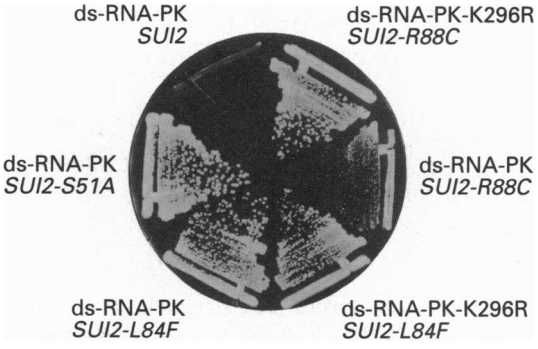

FIG. 3. The *SUI2* suppressor mutations alleviate the toxic effect of human dsRNA-PK on cellular growth rate. Yeast strains H1816, H1817, H1927, and H1928 were transformed with plasmids p1420 and p1421 (8) containing, respectively, wild-type dsRNA-PK or the catalytically inactive mutant dsRNA-PK-K296R, both under the control of a galactose-inducible promoter. Strains containing the indicated *SUI2* alleles and either dsRNA-PK or dsRNA-PK-K296R were streaked on synthetic medium containing 10% galactose (to induce expression of the dsRNA-PK constructs) and incubated for 10 days at 30°C.

between the protein kinase domain and the mutant forms of the eIF-2α substrate molecules that would reduce or eliminate phosphorylation on Ser-51.

The Suppressor *SUI2* Mutations Do Not Reduce Phosphorylation of eIF-2α by GCN2. To address more directly the possibility that the *SUI2* mutations affect the ability of GCN2 to phosphorylate eIF-2α, we examined their effects on the level of eIF-2α phosphorylation *in vivo*. Isoelectric-focusing PAGE was used to resolve eIF-2α isoforms that differ by phosphorylation on Ser-51, and immunoblot analysis was used to visualize these different isoforms. As has been shown (6), the *GCN2^c-E532K,E1522K* allele leads to a relatively high level of phosphorylation independent of amino acid availability that requires Ser-51 in eIF-2α (compare lanes 1 and 2 with 11 and 12 in Fig. 4). The high-level phosphorylation shown in Fig. 4 for the *GCN2^c-E532K,E1522K SUI2* strain is observed in wild-type *GCN2 SUI2* strains only under starvation conditions (2). Replacement of wild-type *SUI2* with the *SUI2-V89I*, *SUI2-L84F*, or *SUI2-I58M* suppressor alleles clearly did not reduce the level of eIF-2α phosphorylation in the presence of *GCN2^c-E532K,E1522K*. In fact, the proportion of phosphorylated eIF-2α increased in these mutants compared with that seen in the corresponding *SUI2* strain, becoming the predominant isoform of eIF-2α. Moreover, the most effective suppressor, *SUI2-L84F*, gave rise to the highest proportion of phosphorylated eIF-2α. These results indicate that these three *SUI2* mutations overcome the toxicity of constitutively activated forms of GCN2 by diminishing the inhibitory effects of phosphorylated eIF-2α on translation initiation, rather than decreasing the proportion of eIF-2α that is phosphorylated. Because the eIF-2α proteins in these three mutants focus at the same position as wild-type eIF-2α, we can also rule out the possibility that the *SUI2* suppressors affect phosphorylation of eIF-2α at other sites, including the potential site of phosphorylation by the cAMP-dependent protein kinase at Ser-90.

In the case of *SUI2-R88C*, the mobility of the eIF-2α protein is shifted toward the acidic end of the gel because the mutation substitutes a positively charged arginine with an uncharged cysteine. The mutation clearly does not abolish eIF-2α phosphorylation; however, it is possible that its weak suppressor phenotype can be accounted for by a reduced efficiency of eIF-2α phosphorylation by GCN2 and dsRNA-PK.

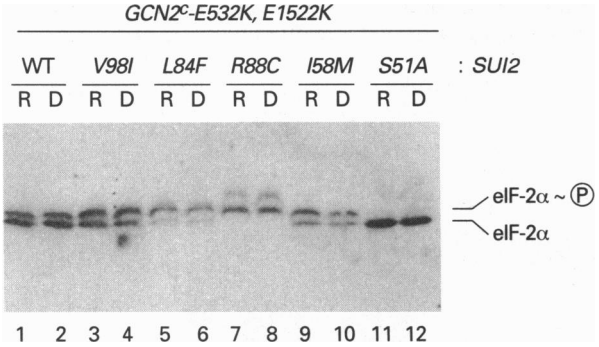

FIG. 4. Isoelectric-focusing gel electrophoresis of eIF-2α from strains carrying *GCN2^c-E532K,E1522K* and different *SUI2* suppressor alleles. Strains H1816, H1817, H1926, H1927, H1928, and H1929 containing *GCN2^c-E532K,E1522K* and the indicated *SUI2* alleles on low-copy-number plasmids were grown under nonstarvation conditions (repressing, R) or under conditions of histidine starvation (derepressing, D) for a total of 6 hr. For derepressing conditions, cultures were supplemented with 3-AT for 1 hr before harvesting. Samples of total cellular protein were separated by isoelectric focusing on a vertical slab gel as described (2) and subjected to immunoblot analysis using polyclonal antiserum specific for yeast eIF-2α and ^{125}I-labeled protein A to visualize immune complexes, all as described (21). WT, wild type.

The *SUI2* Suppressors Overcome the Slow-Growth Phenotype and Derepression of *GCN4* Conferred by a Specific *gcn3^c* Mutation. The characteristics of the *SUI2* suppressors described thus far suggested to us that they eliminate the predicted inhibitory effect of eIF-2α phosphorylation on the ability of eIF-2B to recycle eIF-2·GDP to eIF-2·GTP. We reasoned that if this explanation were correct, the *SUI2* mutations should not suppress mutations in subunits of eIF-2B that impair its function in the absence of eIF-2α phosphorylation. This expectation was borne out for the *gcn3^c-Δ303-305* allele that is missing the last three amino acids of the GCN3 subunit of eIF-2B. The *gcn3^c-Δ303-305* allele conferred slow growth and derepression of *GCN4* (3-AT resistance) in a *gcn2Δ* strain, and these phenotypes were not altered by any of the four *SUI2* suppressor alleles (Table 2 and data not shown). The *gcn3^c-R104K* allele also conferred slow-growth and derepression of *GCN4* in the absence of *GCN2* or Ser-51 on eIF-2α; however, these phenotypes were eliminated by the four *SUI2* suppressor alleles, as shown in Table 2 for *SUI2-V89I* and *SUI2-L84F*. It is interesting that *SUI2-S48A* also overcame the phenotypes of *gcn3^c-R104K*. We showed previously that this *SUI2* mutation partially suppressed the slow-growth phenotype of the *GCN2^c-E532K,E1537G* allele and led to an increase in the extent of eIF-2α phosphorylation (2), thus resembling the four *SUI2* mutations described here. The ability of the *SUI2* suppressors and *SUI2-S48A* to overcome the phenotypes of *gcn3^c-R104K* suggests to us that substitution of Arg-104 in GCN3 affects the interaction between eIF-2 and eIF-2B in a way that mimics the deleterious effect of eIF-2α phosphorylation on the function of eIF-2B.

DISCUSSION

In *S. cerevisiae*, high-level phosphorylation of eIF-2α inhibits general translation initiation and, at low levels, is responsible for translational derepression of the *GCN4* gene (2, 7, 8). Studies of cell-free translation in reticulocyte lysates led to the conclusion that phosphorylation of eIF-2α indirectly inhibits eIF-2 function by impairing the ability of eIF-2B to catalyze guanine nucleotide exchange on eIF-2 after each round of initiation. eIF-2 phosphorylated on the α subunit

Table 2. Allele-specific suppression of the *gcn3^c-R104K* mutation by *SUI2* alleles

SUI2 allele	Plasmid	Colony size of *gcn2Δ* transformants, degree of growth: Vector (YCp50) SD	Vector (YCp50) 3-AT	*GCN3* (Ep69) SD	*GCN3* (Ep69) 3-AT	*gcn3^c-R104K* (Ep305) SD	*gcn3^c-R104K* (Ep305) 3-AT	*gcn3^c-Δ303-305* (Ep319) SD	*gcn3^c-Δ303-305* (Ep319) 3-AT
SUI2	p1097	+++	−	+++	−	+	+	+	+
SUI2-S51A	p1098	+++	−	+++	−	+	+	+	+
SUI2-S48A	p1105	+++	−	+++	−	+++	−	+	+
SUI2-V89I	p1349	+++	−	+++	−	+++	−	+	+
SUI2-L84F	p1350	+++	−	+++	−	+++	−	+	+

Strain H2116 was transformed with the low-copy-number *LEU2* plasmids listed in the second column bearing the *SUI2* alleles indicated in the first column and with the low-copy-number *URA3* plasmids (in parentheses) bearing the *GCN3* alleles (14) indicated across the top. The resulting transformants were tested for growth on SD minimal medium by analyzing the sizes of colonies formed from single cells after incubating 2 days at 30°C and for growth on minimal medium supplemented with 3-AT 3 days after replica-plating patches of cells. Degree of growth is indicated qualitatively by the numbers of plus signs (+++ for wild-type growth) or by a minus sign (little or no detectable growth).

sequesters eIF-2B in an inactive complex and, because eIF-2 is more abundant than eIF-2B, phosphorylation of only a portion of eIF-2α can reduce eIF-2B activity to very low levels (1).

Little is known at the molecular level about how eIF-2 and eIF-2B interact and how this interaction inhibits eIF-2B activity when eIF-2α is phosphorylated. We have undertaken a genetic approach to this problem by isolating mutations in *S. cerevisiae* that overcome the inhibitory effects of constitutively activated forms of the eIF-2α kinase GCN2 on translation initiation. We have shown that a Ser-51 → Ala substitution completely eliminates the phenotypes associated with a high level of GCN2 protein kinase activity in yeast cells (2, 6). By contrast, three of the four mutations described here alter the eIF-2α protein in ways that do not reduce its phosphorylation by GCN2 but, instead, decrease the inhibitory effect of phosphorylated eIF-2 on translation initiation.

There are several ways to explain the ability of these *SUI2* mutations to overcome the toxicity of a hyperactive eIF-2α kinase. Our preferred model is that the mutations alter the interaction between the phosphorylated form of eIF-2 and the eIF-2B complex, such that eIF-2B is no longer inactivated by phosphorylated eIF-2. This result could arise from a weaker physical interaction between phosphorylated eIF-2 and eIF-2B that prevents the formation of a stable inactive complex between the two proteins. The *SUI2* suppressors would not be expected to significantly alter the efficiency of eIF-2 recycling under normal growth conditions when eIF-2α is not being phosphorylated. In accord with the latter, no growth defect was associated with the *SUI2* mutations in *gcn2*Δ strains (Table 2, vector column).

We suggested a similar mechanism to explain the fact that deletion of the *GCN3* gene overcomes the inhibitory effects of eIF-2α hyperphosphorylation by *GCN2^c* protein kinases and by the human kinase dsRNA-PK (8). Recent work indicates that GCN3 is a nonessential subunit of eIF-2B, and the only known function of GCN3 is to mediate the inhibitory effects of phosphorylated eIF-2 on translation initiation (6, 8, 9, 14). On the basis of this result, it was proposed that GCN3 might provide a point of direct contact between eIF-2B and eIF-2α and contribute substantially to the stable interaction between these two complexes that occurs when eIF-2 is phosphorylated. Thus, removal of GCN3 from eIF-2B would have the same effect proposed above for the *SUI2* suppressors in destabilizing the inactive complex between eIF-2B and phosphorylated eIF-2. The fact that we isolated four different suppressor mutations in eIF-2α and none affecting the β or γ subunits of eIF-2 (C.R.V. and A.G.H., unpublished work) supports the idea that contact between eIF-2α and eIF-2B plays a key role in the inhibition of eIF-2B activity. In this view, the region of eIF-2α containing residues 48–89, which encompasses all four *SUI2* suppressor mutations, Ser-48, and the phosphorylation site at position 51, would participate directly in the physical interaction between eIF-2α and eIF-2B.

An alternative mechanism could be proposed in which the *SUI2* mutations suppress the inhibitory effects of eIF-2α phosphorylation by overcoming or reducing the requirement for eIF-2B in recycling eIF-2. For example, the mutations might decrease the affinity of eIF-2 for GDP or increase its affinity for GTP, reducing the need for the guanine nucleotide exchange activity of eIF-2B in translation initiation. One argument against this model is that altering such a fundamental aspect of eIF-2 function would probably be deleterious to the initiation process and, as noted above, the *SUI2* suppressors do not impair cellular growth in wild-type *GCN2* strains. A second argument against this model is that the *SUI2-L84F* allele completely suppresses the toxic effects of extremely high levels of eIF-2α phosphorylation, making it necessary to postulate that this mutation renders eIF-2 completely independent of eIF-2B recycling function. A third argument is that the *SUI2* suppressors do not alleviate the growth defects associated with the *gcn3^c-Δ303-305* allele that appears to impair eIF-2B function independently of eIF-2α phosphorylation (Table 2). Similarly, we found that the *SUI2-L84F* allele does not suppress the growth defects and *GCN2*-independent derepression of *GCN4* associated with reduced-function mutations in two other subunits of eIF-2B, *gcd1-101* and *gcd6-1* (data not shown). These results make it very unlikely that the *SUI2* suppressor mutations decrease the requirement for eIF-2B-catalyzed GDP–GTP exchange on eIF-2.

The fact that the *SUI2* mutations can suppress the *gcn3^c-R104K* mutation, as well as the *GCN2^c* alleles, and that *gcn3^c-R104K* confers slow-growth and derepression of *GCN4* in the absence of eIF-2α phosphorylation could be explained by proposing that a lysine residue at position 104 in GCN3 causes eIF-2B to be sequestered in an inactive complex by nonphosphorylated eIF-2, mimicking the consequences of eIF-2α phosphorylation in wild-type *GCN3* cells. We suggested above that a domain of eIF-2α containing residues 48–89 physically interacts with GCN3. The suppression of *gcn3^c-R104K* by the *SUI2* alleles could indicate that the region surrounding Arg-104 in GCN3 is directly involved in this postulated interaction between GCN3 and eIF-2α. Testing this model will require *in vitro* analysis of the effects of *SUI2* suppressor mutations on the stability and recycling activity of the eIF-2·eIF-2B complex in the presence and absence of eIF-2α phosphorylation. Biochemical studies on these *SUI2* alleles and on mutations in different subunits of eIF-2B, which similarly diminish its sensitivity to eIF-2α phosphorylation, should lead us to a detailed molecular model for the regulation of eIF-2B function by phosphorylated eIF-2.

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