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Mutations Causing Childhood Ataxia with Central Nervous System Hypomyelination Reduce Eukaryotic Initiation Factor 2B Complex Formation and Activity

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Childhood ataxia with central nervous system hypomyelination (CACH), or vanishing white matter leukoencephalopathy (VWM), is a fatal brain disorder caused by mutations in eukaryotic initiation factor 2B (eIF2B). eIF2B is essential for protein synthesis and regulates translation in response to cellular stresses. We performed mutagenesis to introduce changes equivalent to 12 human CACH/VWM mutations in three subunits of the equivalent factor from yeast (*Saccharomyces cerevisiae*) and analyzed effects on cell growth, translation, and gene expression in response to stresses. None of the mutations is lethal or temperature sensitive, but almost all confer some defect in eIF2B function significant enough to alter growth or gene expression under normal or stress conditions. Biochemical analyses indicate that mutations analyzed in eIF2Bα and -ε reduce the steady-state level of the affected subunit, while the most severe mutant tested, eIF2Bβ^{V341D} (human eIF2B$^{\beta V316D}$), forms complexes with reduced stability and lower eIF2B activity. eIF2Bδ is excluded from eIF2Bβ^{V341D} complexes. eIF2B$^{\beta v341D}$ function can be rescued by overexpression of eIF2Bδ alone. Our findings imply CACH/VWM mutations do not specifically impair responses to eIF2 phosphorylation, but instead cause protein structure defects that impair eIF2B activity. Altered protein folding is characteristic of other diseases, including cystic fibrosis and neurodegenerative disorders such as Huntington, Alzheimer's, and prion diseases.

Childhood ataxia with central nervous system hypomyelination (CACH), also called vanishing white matter (VWM) leukoencephalopathy (Online Mendelian Inheritance in Man [OMIM] no. 603896 at http://www.ncbi.nim.nih.gov/entrez/dispomim.cgi) is an inherited autosomal-recessive disease first described in 1994 (42). Clinically, CACH/VWM is a chronic progressive disorder mainly affecting children. Deterioration often follows acute fever or head trauma—periods of stress. The disorder primarily affects glial cells of the brain, with oligodendrocytes described as abnormal and "foamy" in appearance (52). There are also reports of increased number and density of oligodendritic cells and a reduced number of astrocytes (17). The disorder is usually diagnosed following magnetic resonance imaging in which the signal intensity of at least part of the white matter is altered and resembles cerebrospinal fluid, hence the name VWM (34). The principal role of oligodendrocytes is to form myelin sheaths around neurons. Much brain development, including myelin formation, occurs after birth. The symptoms and progression of the disease are therefore variable and dependent on age of onset, with early-onset forms leading to more severe symptoms and earlier morbidity (14, 15).

CACH/VWM is one of several disorders known to affect white matter, many of which have had a genetic lesion identified. These analyses reveal that a variety of defects cause white matter abnormalities, including mutated myelin proteins, lipids, and accumulation of amino acid derivatives (32). Recently the genetic cause of CACH/VWM was identified when a large number of missense mutations were found in each of the genes encoding the five subunits of eukaryotic translation initiation factor 2B (eIF2B) (34, 47). This is the first human disease shown to affect a protein synthesis factor directly. eIF2B functions in translation as a guanine nucleotide exchange factor (GEF) to promote release of GDP from its substrate eIF2 and formation of an active eIF2-GTP complex. eIF2-GTP then binds aminoacylated initiator methionyl-tRNA (Met-tRNA$_i^{Met}$), forming a ternary complex (TC) (9). TC is essential for each translation initiation event. TC formation is regulated by controlling eIF2B function, and this is likely important in CACH/VWM.

Four protein kinases have been described that phosphorylate eIF2 on its α subunit at Ser-51 [eIF2(αP)]. Each kinase reacts to different cellular stress conditions. GCN2 responds to amino acid starvation (28); PEK/PERK counters damage caused by unfolded proteins in the endoplasmic reticulum (ER); PKR is activated by double-stranded RNA in response to viral infection, and HRI is regulated by heme levels in reticulocytes (9). eIF2(αP) reduces the activity of eIF2B, with the two proteins forming a nonproductive eIF2(αP)-eIF2B complex (37). Because eIF2 is more abundant than eIF2B, even a low eIF2(αP) percentage can reduce eIF2B activity and therefore lower TC levels. Lower TC levels have opposing effects: general protein synthesis is reduced, but translation of stress responsive genes is enhanced. The best-characterized example of this gene-specific control is the activation of the translation of *GCN4* mRNA by ~10-fold following amino acid starvation in *Saccharomyes cerevisiae*. *GCN4* translation is dependent on short upstream open reading frames (uORFs) in

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the *GCN4* mRNA 5′ leader, which limit the flow of scanning ribosomes to the *GCN4* ORF under nonstress conditions. Upon limitation for one or more amino acids, Gcn2p is activated, and the resulting reduced TC levels mediate ribosome scanning past the inhibitory uORFs and allow elevated translation of the *GCN4* ORF (29). Gcn4p protein then activates transcription of multiple genes (>500), including 73 amino acid biosynthetic genes (36). The mammalian transcriptional activator *ATF4* is similarly controlled by uORFs following activation of mGCN2 or PERK (25). This also alters transcription of many stress response genes (26).

The activity of mammalian eIF2B can be controlled directly in response to insulin signaling via reduced glycogen synthase kinase 3 phosphorylation of the eIF2Bε subunit (50). It was also recently found that mutation of eIF2Bγ increases the sensitivity of yeast to fusel alcohols such as butanol (3). Fusel alcohols are produced by yeast following catabolism of amino acids, and their presence may signal nutrient scarcity (12).

Functions for each of the five subunits of eIF2B have been assigned in the main from molecular genetic and biochemical studies of the yeast factor. Four of the five subunits (β-ε) are essential for yeast growth, while the smallest (α) is only required for response to amino acid starvation and activation of *GCN4* translation. Nonlethal mutations in all five yeast subunits that reduce eIF2B activity have been described. The eIF2Bα, -β, and -δ subunits share sequence similarity. All three act together, forming a regulatory subcomplex that mediates the inhibition of eIF2B function in response to eIF2(αP) (53). Single missense mutations in any one of these three eIF2B subunits can prevent the inhibitory function of eIF2(αP) (37, 38). This regulatory complex binds the phosphorylated eIF2α subunit [eIF2α(P)] directly, and regulatory mutations in eIF2Bβ act to reduce the eIF2B-eIF2α(P) affinity (33). In contrast, the γ and ε subunits of eIF2B are required for the catalytic function of nucleotide exchange. The C-terminal ~200 amino acids of eIF2Bε harbor the "catalytic center" of the enzyme, while the remainder of these subunits are proposed to function in subunit-subunit interactions (20, 21, 37).

Yeast provides excellent tools to study eIF2B structure, function, and regulation in response to stress via eIF2(αP) and fusel alcohols. Also, yeast is the only organism in which an extensive panel of mutations have been characterized previously. We therefore set out to analyze the effects of mutations equivalent to the human mutations causal for CACH/VWM on the ability of yeast to grow and respond to various cellular stresses known to alter eIF2B function. Our aim was to uncover what the molecular defect or defects in eIF2B are responsible for this devastating and fatal human disorder.

MATERIALS AND METHODS

Growth conditions and genetic methods. *S. cerevisiae* was grown in standard laboratory media. Amino acid and nucleotide supplements were used to maintain plasmid selection as appropriate (43). 3-Aminotriazole (3AT) (10), butanol (3), ethanol, or azetidine-2-carboxylic acid (AZC) (46) was added where indicated in the figure legends. Drug gradient plates were made as described previously (13). For yeast spot analysis, cell cultures were grown in SD to an A_{600} of 0.3. Fivefold serial dilutions were performed, and the appropriate media were each spotted with 3 μl of each dilution. For gradient plates, cultures were grown to an A_{600} of 0.125 and diluted to 0.0625. Three microliters of this dilution was applied as spots across the gradient. The yeast strains used were made by transformation of the strains described in Table 1 with plasmids by the lithium acetate method (19). Plasmid shuffling used 5-fluoroorotic acid (4).

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
BJ1995	*MAT*α *leu2 trp1 ura3-52 gal2 pep4-3 prb1-1122*	31
GP3153	*MAT***a** *leu2-3 leu2-112 trp1-Δ63 ura3-52 gcn3::LEU2*	38
H1725	*MAT*α *leu2-3 leu2-112 ura3-52 gcd7-201*	5
H1727	*MAT*α *leu2-3 leu2-112 ura3-52*	5
H1794	*MAT*α *leu2-3 leu2-112 ura3-52 gcn2::LEU2 gcd7-201*	5
H2217	*MAT***a** *leu2-3 leu2-112 trp1-Δ63 ura3-52 gcd7Δ::hisG GCN4-lacZ* at *TRP1* pJB99[*GCD7 URA3*]	49
H2218	*MAT***a** *leu2-3 leu2-112 trp1-Δ63 ura3-52 gcn2Δ gcd7ΔhisG GCN4-lacZ* at *TRP1* pJB99[*GCD7 URA3*]	49
KAY16	*MAT*α *leu2-3 leu2-112 ura3-52 ino1 gcn2Δ::hisG gcd6Δ HIS4-lacZ* at *ura3-52* pJB5[*GCD6 URA3*]	21

Plasmids. The plasmids used are summarized in Table 2. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) to introduce mutations equivalent to human CACH/VWM mutations into the coding regions of eIF2Bβ (*GCD7*) in plasmid p1558, creating plasmids pAV1747-1751; eIF2Bε (*GCD6*) in plasmid pJB102, creating pAV1739-1745; and eIF2Bα (*GCN3*) in plasmid pAV1769, creating plasmid pAV1778. Details of all site-directed mutagenesis oligonucleotides used will be provided on request. All constructs were verified by DNA sequence analysis.

Other plasmids were made by standard subcloning procedures (41). The eIF2Bε (*GCD6*) plasmid pAV1754 was made by deleting *GCD1* sequences from the high-copy-number plasmid p1873 (11) by using *Xho*I and religating to create an *Xho*I-*Spe*I *GCD6* fragment in pRS425. eIF2Bε mutant plasmids pAV1754 to -1761 were made by subcloning each mutant *GCD6* from plasmids pAV1739 to -1745 on *Not*I-*Xho*I fragments into similarly digested pRS425. The eIF2Bα (*GCN3*) plasmid pAV1769 was created by subcloning the 1.9-kb *Spe*I-*Bam*HI fragment from Ep69 (23) into similarly digested pSR316. pAV1136 was constructed exactly as described for pAV1139 and pAV1140 (37), except that wild-type eIF2Bβ (*GCD7*) derived from p1558 was used (49). pAV1874 was constructed by digesting pAV1747 with *Xho*I and *San*DI to produce a 350-bp restriction fragment containing the eIF2Bβ^{V341D} (*gcd7*-V341D) mutation. This restriction fragment was ligated into similarly digested pAV1136.

Immunoblotting. Proteins were resolved on 12.5% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and visualized by immunoblotting with specific antisera as described previously (11). Detection was by enhanced chemiluminescence (Amersham) with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibodies.

Determination of β-galactosidase activity. The β-galactosidase activity of *GCN4-lacZ* and *HIS4-lacZ* reporters was determined as described previously (10).

In vitro nucleotide exchange assays. In vitro nucleotide exchange assays using eIF2-[^{3}H]GDP as a substrate with 100-fold excess unlabeled GDP and FLAG-purified eIF2B or eIF2B$^{\beta V341D}$ were performed at 10°C as described previously (2).

Protein purification. Yeast strain BJ1995 transformed with plasmids overexpressing all five subunits of FLAG-tagged eIF2B (pAV1136 + pTK11.1) or eIF2B$^{\beta V341D}$ (pAV1784 and pTK11.1) were grown in 2.4 liters of SC to an A_{600} of 3.5. Cultures were harvested at 4°C by centrifugation and washed twice in ice-cold water containing 1 mM phenylmethlysulfonyl fluoride (PMSF). Cell pellets were resuspended in 2 cell volumes of buffer LB-500 (500 mM KCl, 20 mM Tris-HCl [pH 7.5], 3 mM $MgCl_2$, 0.1% [vol/vol] Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, 1-μg/ml leupeptin, 0.7-μg/ml pepstatin, 5-μg/ml aprotinin, 1× complete EDTA-free protease inhibitor cocktail tablet [Roche]). This cell suspension was dripped into liquid nitrogen to form yeast "beads." Beads were ground to a fine powder with a mortar and pestle in liquid nitrogen and stored at −80°C.

Ground yeast powder was resuspended in buffer LB-500 at 4°C, and all sub-

TABLE 2. Plasmids used in this study

Plasmid	Copy no.[a]	Gene/allele[b]	Source or reference
p1558	*CEN*	*GCD7 LEU2*	49
pAV1747	*CEN*	*gcd7*-V341D *LEU2*	This study
pAV1748	*CEN*	*gcd7*-G354V *LEU2*	This study
pAV1749	*CEN*	*gcd7*-E239G *LEU2*	This study
pAV1751	*CEN*	*gcd7*-K300R *LEU2*	This study
pJB102	*CEN*	*GCD6 LEU2*	5
pAV1739	*CEN*	*gcd6*-V57G *LEU2*	This study
pAV1740	*CEN*	*gcd6*-I90F *LEU2*	This study
pAV1741	*CEN*	*gcd6*-R323P *LEU2*	This study
pAV1742	*CEN*	*gcd6*-G369V *LEU2*	This study
pAV1743	*CEN*	*gcd6*-I413A *LEU2*	This study
pAV1744	*CEN*	*gcd6*-R284H *LEU2*	This study
pAV1745	*CEN*	*gcd6*-W618R *LEU2*	This study
pRS425	2μm	*LEU2*	6
pAV1754	2μm	*GCD6 LEU2*	This study
pAV1755	2μm	*gcd6*-V57G *LEU2*	This study
pAV1756	2μm	*gcd6*-I90F *LEU2*	This study
pAV1757	2μm	*gcd6*-R284H *LEU2*	This study
pAV1758	2μm	*gcd6*-R323P *LEU2*	This study
pAV1759	2μm	*gcd6*-G369V *LEU2*	This study
pAV1760	2μm	*gcd6*-I413A *LEU2*	This study
pAV1761	2μm	*gcd6*-W618R *LEU2*	This study
pAV1769	*CEN*	*GCN3 URA3*	This study
pAV1778	*CEN*	*gcn3*-N209Y *URA3*	This study
pRS316	*CEN*	*URA3*	44
pAV1136	2μm	*GCD7 GCD2 GCN3 URA3*	37
pAV1784	2μm	*gcd7*-V341D *GCD2 GCN3 URA3*	This study
pTK1-11	2μm	*GCD1*-FLAG-HIS_6 *GCD6 LEU2*	21
pJB99	*CEN*	*GCD7 URA3*	5
p2297	2μm	*GCD2 URA3*	51

[a] *CEN* indicates low-copy-number plasmids, and 2μm indicates high-copy-number plasmids.
[b] *GCD7* encodes eIF2Bβ, *GCD6* encodes eIF2Bε, *GCN3* encodes eIF2Bα, *GCD1* encodes eIF2Bγ, and *GCD2* encodes eIF2Bδ.

sequent steps were maintained at 4°C. Crude lysates were cleared by centrifugation at 30,000 × *g* for 30 min to produce a starting extract. M2 FLAG affinity resin (Sigma) was preequilibrated by washing three times in LB-500. Extracts were added to 250 μl of 50% M2 resin slurry and incubated for 2 h with rolling. FLAG resin was collected by centrifugation and washed three times in buffer WB-100, which is identical to LB-500, except 5% (vol/vol) glycerol, 100 mM KCl, and 5 mM NaF were added and $MgCl_2$ was omitted. Resin was then incubated for 30 min with rolling in WB-100 containing 3× FLAG peptide (Sigma) to a final concentration of 4 μg/ml to competitively elute the retained proteins. Eluted proteins were stored at −80°C.

Immunoprecipitation of eIF2B from WCE. eIF2B was immunoprecipitated from whole-cell extract (WCE) as follows. Yeast strains derived from H2217 containing wild-type or mutant eIF2Bβ subunits were grown in 500 ml of SC to an A_{600} of 1.0. Cells were harvested by centrifugation and washed twice in ice-cold water containing 1 mM PMSF. Cell pellets were resuspended in LB-150 (LB-150 is identical to LB-500, except that KCl, $MgCl_2$, and β-mercaptoethanol were replaced with 150 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol [DTT], respectively), and extracts were prepared by grinding in liquid nitrogen as described above. All subsequent steps were performed at 4°C.

Protein A-Sepharose beads (Amersham Biosciences) were washed three times in LB-150 without protease inhibitors and incubated with anti-Gcd6p polyclonal serum (rabbit) for 1 h. Following three further washes, cell extracts (1 mg) were added to a 50% slurry of antibody-conjugated Sepharose beads and incubated for 2 h. Beads were collected by centrifugation at 400 × *g*, and the supernatant fraction was removed. Beads were washed three times in LB-150 (containing inhibitors). Pellet fractions were eluted by boiling in SDS-PAGE loading buffer. Detection following immunoblotting employed HRP-linked protein A (Sigma) in place of HRP-conjugated antirabbit secondary antibody.

Polyribosome distribution on sucrose gradients. Yeast strains were grown in 100 ml of YPD to an A_{600} of 0.7. Cycloheximide was added to 50 μg/ml, and the cultures were placed on ice. For butanol experiments, the culture was split, and 1% (vol/vol) butanol was added to one-half (50 ml) of the culture, which was then incubated for 10 min at 30°C prior to cycloheximide addition. Methods for cell harvesting and sucrose density gradient fractionation (performed with 15 to 50% sucrose gradients on an ISCO gradient fractionator) were described previously (7, 16).

RESULTS

Selection of CACH/VWM mutations for analysis. Leegwater and colleagues (33) described four missense mutations in human eIF2Bβ and 14 mutations in eIF2Bε that are associated with CACH/VWM. For each patient studied, both alleles of the same gene were affected: either two missense mutations were present, or a missense mutation was combined with a nonsense or frameshift mutation that would prematurely terminate the ORF (Fig. 1A). It was concluded that missense mutations were responsible for the disorder and that the disease was recessive. It was also noted that the missense mutations were spread throughout the coding regions of both subunits.

We performed multiple sequence alignments using ClustalX (45) and BLASTp (1) tools to determine how closely related the yeast and human sequences were in the regions where the residues are altered in CACH/VWM. This revealed that several CACH/VWM mutations were in regions of high sequence conservation, while others were not (Fig. 1 and data not shown). Interestingly, R113, the residue found most commonly mutated in CACH/VWM patients (eIF2Bε^{R113H}), is not conserved in all other mammals. *Mus musculus* (mouse), for example, already has a histidine at the equivalent residue (H109)! We selected mutations to study where the yeast and human residues were identical or, in two instances, where our alignments indicated a similar residue in both species, but where the CACH/VWM mutation substituted a nonconserved residue (Fig. 1A). Secondary structure prediction software (PSIPRED) (35) was used to analyze the human and yeast sequences. We examined whether introducing the mutations selected would alter these predictions. Eight of the 12 residues affected by CACH/VWM mutants we selected gave high confidence scores for the secondary structure prediction, and none of these predictions was altered by the single-amino-acid change in either species. Only secondary structures around human/yeast eIF2B εV73/57, εR299/284, βK273/300, and αN208/209 were predicted with low confidence. Although highly speculative, this computational analysis suggests the mutations do not act by destabilizing local secondary structure.

We also noted that previously described mutations in yeast eIF2Bβ that reduce the sensitivity of eIF2B to regulation by phosphorylation of eIF2α are localized in residues close to those identified in CACH/VWM (36) (Fig. 1C, lowercase mutations). Following subsequent identification of CACH/VWM mutations in the α, β, and δ subunits of eIF2B (47), we found that the human *EIF2B1*-N208Y mutation alters a residue in a region highly conserved with yeast (Fig. 1D). This mutation is close to the yeast eIF2Bα-E199K (*GCN3*^{c}-E199K) mutation, which reduces eIF2B activity rather than causing a specific defect in the response to eIF2(αP) (22).

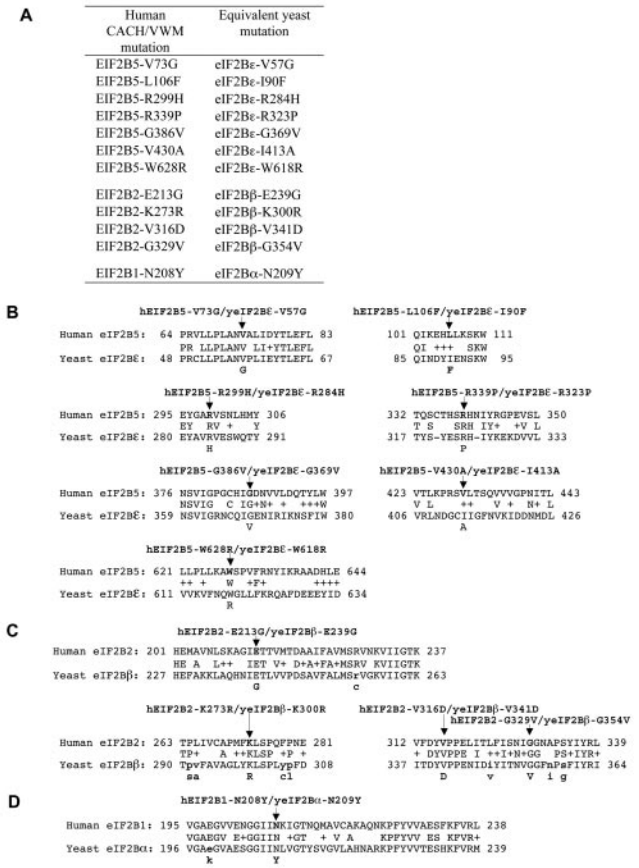

FIG. 1. CACH/VWM mutations analyzed. (A) Residues mutated in human eIF2B genes and the equivalent substitutions in yeast. V73G indicates valine is the wild-type residue at position 73 and is substituted for glycine in the mutant. (B to D) Segments of BLASTp amino acid sequence alignments between human and yeast eIF2B subunits eIF2Bε (B), eIF2Bβ (C), and eIF2Bα (D). Human residues equivalent to those mutated in this study are indicated with an arrow and shown in boldface, with the new residue indicated below the sequence in uppercase. Mutations in yeast eIF2B that were characterized previously are shown in boldface and lowercase (C and D only).

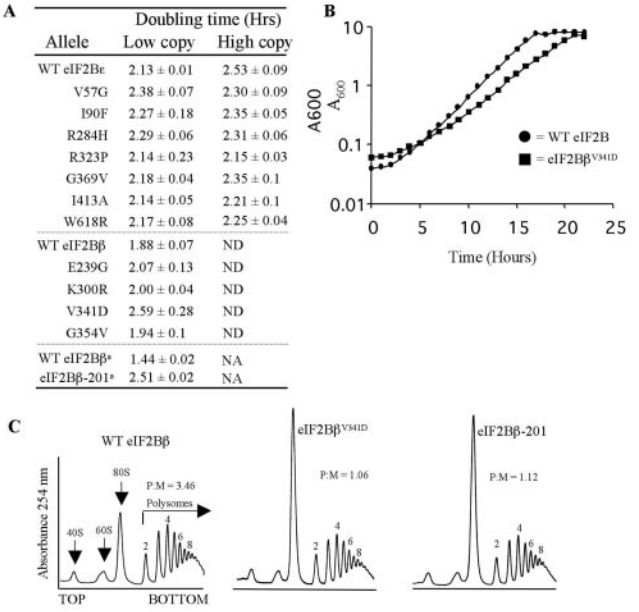

FIG. 2. eIF2Bβ^{V341D} exhibits a reduced growth rate and a defect in global translation. (A) The doubling times of strains containing the indicated alleles were determined in YPD liquid medium during exponential growth. CACH mutant strains were derived from KAY16 (eIF2Bε alleles) or H2217 (eIF2Bβ alleles). [a], for purposes of comparison, the doubling time for the eIF2Bβ-201 strain (H1725) and its isogenic parent (H1727) are shown. High copy indicates 2μm plasmid-borne alleles. WT, wild type; ND, not determined; NA, not applicable (B) Growth curves of wild-type and isogenic eIF2Bβ^{V341D} mutant strains used to generate the doubling time shown in panel A. (C) Polysome profile analyses of wild-type eIF2B, eIF2Bβ^{V341D} (in strain H2217), and eIF2Bβ-201 (strain H1793) mutants cultured in YPD medium at 30°C. 40S, 60S, 80S, and polyribosome peak positions are indicated. The polysome/monosome ratio (P:M) was quantified for each trace by using NIH Image v1.61 software.

CACH/VWM mutations are viable, but eIF2Bβ-V341D (human *EIF2B2*-V316D) has a significant defect in eIF2B function in general translation initiation. The mutations listed in Fig. 1A were constructed by site-directed mutagenesis of plasmid-borne yeast genes and introduced into yeast strains with deletion of the endogenous subunit so that the mutated gene was under control of its own promoter on a low-copy-number plasmid as the only source of that subunit. We found that all yeast strains created were viable, indicating that none of the mutations caused a lethal reduction in an essential function of eIF2B. This must also be true for the human CACH/VWM mutations, because they are not embryonic-lethal mutations.

When the growth rate of each mutant strain was examined carefully on solid and in liquid medium, only one mutant allele (eIF2Bβ-V341D) caused a significantly reduced growth rate (Fig. 2A and B and see Fig. 4B). Human CACH/VWM is brought on by fever, and some previously described yeast eIF2B mutations are temperature sensitive (24). We therefore undertook further tests designed to reveal any temperature-dependent effects. No significant defects were found (data not shown).

To examine the effects of these mutations on global translation initiation, polysome profile analysis was performed to assess the proportion of ribosomes undergoing active translation. A defect in translation initiation is typically identified by an increase in the 80S monosome peak and a decrease in the proportion of mRNAs with two or more ribosomes attached (polysomes). This analysis was performed on all seven eIF2Bε and four eIF2Bβ mutants (data not shown). In agreement with the growth rate analyses, only eIF2Bβ^{V341D} showed a profile significantly different from that of the wild-type control (Fig. 2C). The eIF2Bβ^{V341D} profile has reduced polysomes and elevated 80S monosomes typical of mutations that reduce global translation initiation. The growth rate defect and polysome profile are almost identical to those of a previously described yeast eIF2Bβ mutant- the *gcd7-201* allele, here called eIF2Bβ-201 (5) Fig. 2A and C), confirming that eIF2Bβ^{V341D} has a global translation defect.

eIF2Bα and eIF2Bε mutations have reduced steady-state protein levels. Next we asked whether the expression level of any of the mutated proteins was altered, because this may contribute to a mutant phenotype. Immunoblotting with antiserum specific for subunits of eIF2B was performed on extracts

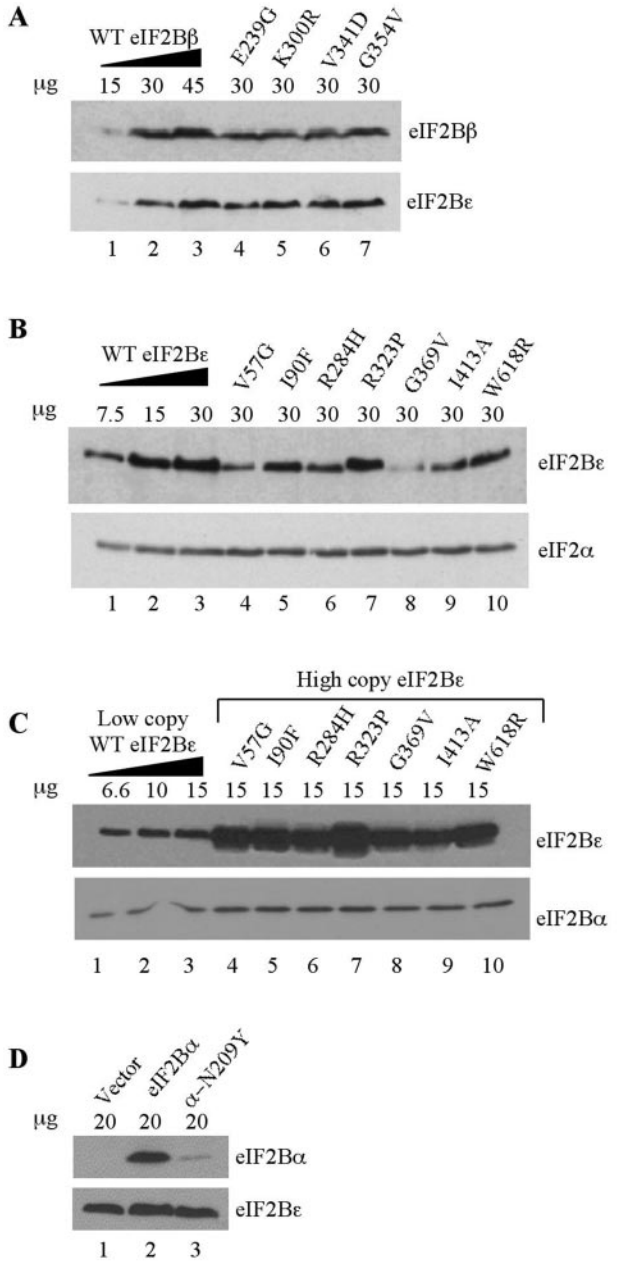

FIG. 3. eIF2Bε and eIF2Bα CACH/VWM mutants reduce steady-state subunit expression levels. Extracts from cells containing the indicated eIF2Bβ (A), eIF2Bε (B and C), or eIF2Bα (D) alleles were analyzed by SDS-PAGE and immunoblotting with the indicated subunit-specific polyclonal antibodies. The amount of cell protein loaded in each lane is indicated in micrograms. For the experiment in panel C, cells contained high-copy-number (H/C) plasmids overexpressing the indicated mutant. WT, wild type.

from cells bearing each allele as the sole source of that subunit. We found that there were no significant differences in the expression levels of all four eIF2Bβ mutations analyzed (Fig. 3A and see Fig. 7D). This indicated that poor expression was not responsible for the growth and translation defects of eIF2Bβ^{V341D} cells. However, in contrast to these results, we observed reproducible reductions in the levels of protein present in cell extracts from six of the seven eIF2Bε mutants (Fig. 3B, compare lane 3 with lanes 4 to 10) and the one eIF2Bα mutant studied (Fig. 3D). Consistent with previous results, reduction in the levels of the mutated subunit did not affect expression of other eIF2B subunits, but must reduce levels of active five-subunit eIF2B complexes in these cells.

CACH/VWM mutations cause defects in gene-specific translational control. To examine the effects of CACH/VWM mutations on gene-specific translational control, the response of CACH/VWM mutant cells to amino acid starvation was assessed. Starvation for one or more amino acid activates the protein kinase Gcn2p that phosphorylates eIF2 and inhibits eIF2B activity. eIF2B mutants with lower eIF2B activity derepress *GCN4* translation even in the absence of a starvation signal and are called *gcd*$^-$ mutations in yeast. In contrast, mutations that reduce the sensitivity of eIF2B to eIF2(αP) fail to derepress *GCN4* translation even in the presence of a starvation signal (*gcn*$^-$ mutations). A *GCN4-lacZ* reporter was used to monitor translational control under normal or amino acid starvation conditions in eIF2Bβ strains. The drug 3-aminotriazole (3AT), an inhibitor of the histidine biosynthesis enzyme encoded by *HIS3*, was used to impose a histidine limitation (10). eIF2Bβ^{V341D} exhibited a significant difference from the wild type (Fig. 4A). This mutant revealed high-level translation of a *GCN4-lacZ* reporter even in the absence of amino acid starvation, similar to the previously described eIF2Bβ-201 allele (5). This result was confirmed with assays to measure growth in the presence of 3AT. Normally *GCN2* is required for growth in the presence of 3AT; however, as seen from the results shown in Fig. 4B, eIF2Bβ^{V341D} and the eIF2Bβ-201 mutant grew on medium containing 3AT when *GCN2* was deleted (*gcn2*Δ). Finally, these two mutants also conferred the ability to grow in the presence of elevated levels of the toxic imino acid analog of proline, AZC. AZC is incorporated into protein competitively with proline, inducing protein malfolding (46). Mutations in eIF2B that confer constitutively high *GCN4* expression synthesize more proline and grow in the presence of AZC.

Responses of eIF2Bε mutant strains to amino acid starvation were also assessed. The yeast strain used has an integrated *HIS4-lacZ* reporter. *HIS4* transcription is regulated by *GCN4*, so it indirectly monitors Gcn4p activity. As with the *GCN4-lacZ* reporter, β-galactosidase activity inversely correlates with eIF2B function. Three mutants have a significant defect in eIF2B function: V57G (human V73G), R284H (human R299H), and W618R (human W628R) (Fig. 5A). These mutations also have enhanced *GCN2*-independent growth in the presence of 3AT and AZC (Fig. 5B).

It was surprising that most eIF2Bε mutations reduced protein levels, but only three appeared to significantly reduce eIF2B activity in vivo. This suggests that yeast cells have excess eIF2B capacity under the growth conditions tested, and only those mutations that reduce eIF2B function below a threshold activate *GCN4* expression. A greater reduction in eIF2B function is required to alter growth rate.

We thought that overexpression of the mutant subunit should correct the reduced protein levels. So we transferred the eIF2Bε alleles to high-copy-number plasmids. Western blotting confirmed that the overexpressed eIF2Bε mutant proteins were present in excess, so normal levels of five-subunit eIF2B could form in these cells (Fig. 3C). Overexpression of

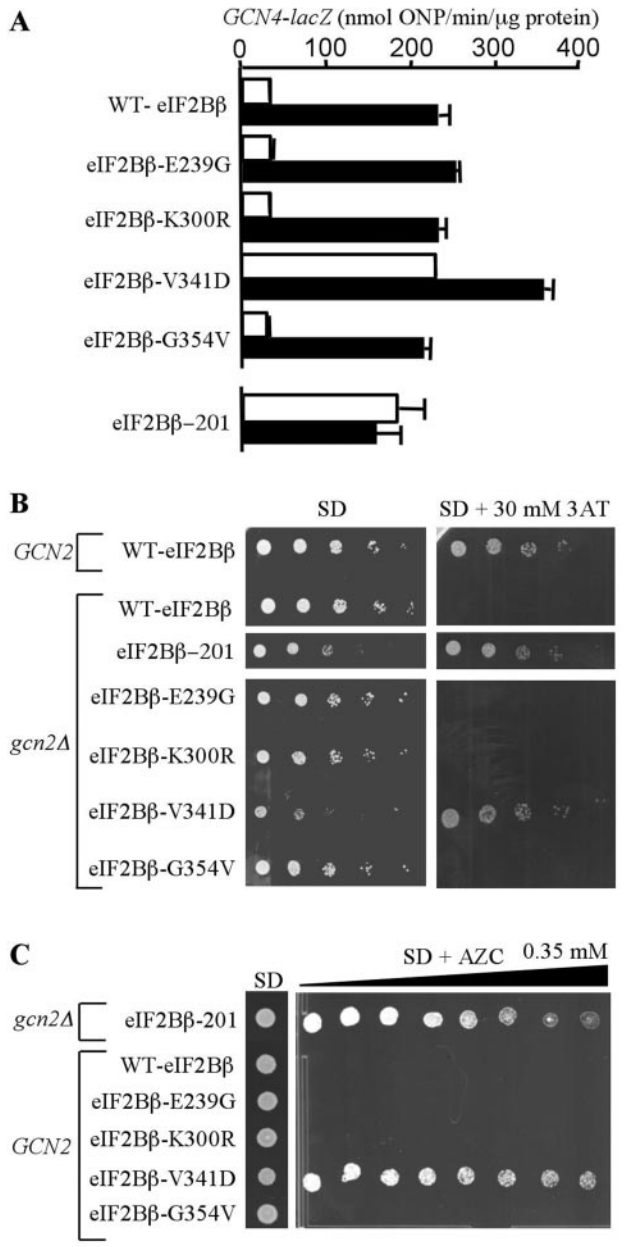

FIG. 4. Gcn4p activity is elevated in eIF2Bβ^{V341D}. (A) *GCN4-lacZ* reporter gene activity of eIF2Bβ CACH/VWM mutants under basal (nonstarvation) conditions (open bars) and under starvation conditions imposed by 10 mM 3AT (solid bars) transformed into *GCN2* strain H2218. The eIF2Bβ-201 control strain H1727 transformed with p180 (5) was also assayed. WT, wild type. (B) Serial dilution of wild-type eIF2B (*GCN2* strain H2218 and *gcn2*Δ strain H2217), eIF2Bβ-01 (*gcn2*Δ, strain H1794), and eIF2Bβ CACH/VWM mutants (*gcn2*Δ in strain H2217) on SD agar ± 30 mM 3AT. (C) Growth of eIF2Bβ-0 (*gcn2*Δ, strain H1794) and eIF2Bβ (*GCN2*, strain H2218) CACH/VWM mutants on SD agar and on the same medium containing a linear gradient of AZC to a maximum concentration of 0.35 mM.

the wild-type eIF2Bε subunit altered *GCN4* expression, as evidenced by increased resistance to 3AT (Fig. 5C, compare the top and second rows). This result indicates that excess eIF2Bε impairs eIF2B function sufficiently to activate *GCN4* expression. The mechanism for this is not known. Perhaps the excess eIF2Bε acts independently to interfere with five-subunit eIF2B function. Alternatively, the excess subunit may promote the formation of eIF2B subcomplexes (37), thereby reducing the levels of functional five-subunit eIF2B. No significant growth defect was observed in cells everexpressing mutated eIF2Bε subunits (Fig. 2A). In addition, we found that the growth differences on 3AT medium evident for non-overexpressed eIF2Bε mutants were largely eliminated, when compared with overexpressed wild-type eIF2Bε. R323P (human R339P) was the only exception, which was now more resistant to very high 3AT concentrations (Fig. 5C). These experiments show that mutations responsible for CACH/VWM can alter expression of translationally controlled genes and their downstream targets in the amino acid starvation response pathways. These experiments suggested that eIF2Bε mutations simply act by lowering expression levels, so to assess whether the overexpressed eIF2Bε mutations had any other defect, we analyzed sensitivity to butanol.

CACH/VWM alleles have increased sensitivity to fusel alcohols. Recently, a yeast eIF2Bγ mutation was described that did not alter cell growth or responses to amino acid starvation, but instead conferred enhanced sensitivity to fusel alcohols such as butanol: eIF2Bγ^{P180S} (3). To assess whether any of the CACH/VWM mutations behave in a similar way, we assessed this response next.

Sensitivity of eIF2B mutations to fusel alcohols has been characterized by a rapid inhibition of general translation initiation, as revealed by polysome profile analysis after a 10-min treatment with butanol combined with a reduced rate of colony formation on solid medium containing butanol (3). We used these two diagnostic tests to screen our panel of CACH/VWM mutations for altered sensitivity to butanol and, as a control, ethanol. We found that eIF2Bβ^{V341D} cells were sensitive to butanol, but not ethanol. This confirms specificity of the alcohol response to eIF2B and shows it is not due to other nonspecific effects of alcohols on yeast (39). Similarly, three eIF2Bε mutants showed butanol-specific growth sensitivity even when overexpressed: I90F, R284H, and W618R (Fig. 6A). The most severely affected mutant was eIF2Bε^{W618R} (human W628R) (Fig. 6A and B). Polysome profile analysis following a 10-min butanol treatment confirmed that global translation initiation was inhibited in eIF2Bε^{W618R} mutant cells to a level similar to that observed for the eIF2Bγ^{P180S} mutation examined previously (3) (Fig. 6C).

In summary, these analyses reveal that eIF2Bβ^{V341D} and eIF2Bε^{R284H} and eIF2Bε^{W618R} cause altered responses to both amino acid starvation and butanol addition. However, eIF2Bε^{I90F} is sensitive only to butanol, and eIF2Bε^{V57G} and eIF2Bε^{R323P} only have altered amino acid responses.

eIF2Bβ^{V341D} reduces activity and stability of the eIF2B complex. Results described to this point are consistent with the idea that eIF2Bβ^{V341D} mutant cells have significantly-reduced eIF2B activity. To test this directly, we employed a FLAG epitope-tagged system to rapidly purify wild-type and mutant eIF2B complexes (eIF2B$^{\beta V341D}$) from cells overexpressing all five subunits of eIF2B in a single step (Fig. 7A). These proteins were then assayed for eIF2B activity, where the ability of eIF2B to displace radiolabeled GDP from preformed eIF2-GDP complexes was determined. Assays were performed at two different eIF2 (substrate)/eIF2B (enzyme) ratios. Increas-

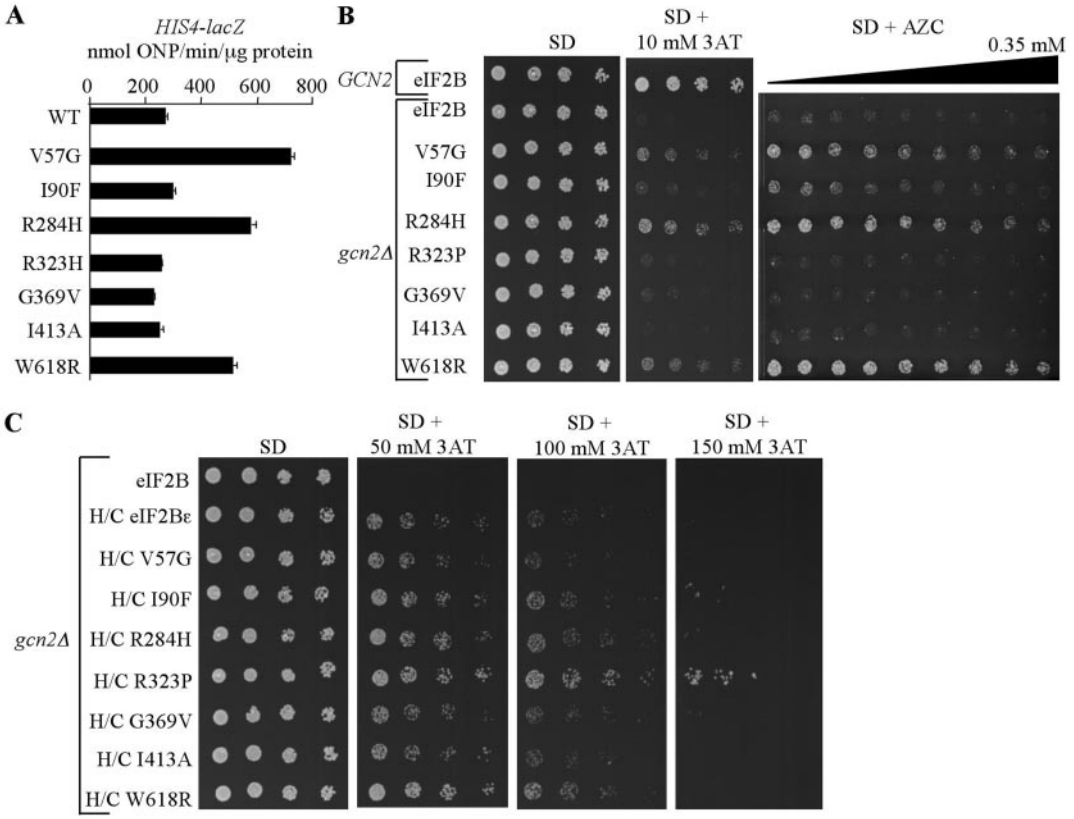

FIG. 5. Gcn4p activity is elevated in some eIF2Bε mutants. (A) *HIS4-lacZ* reporter activity for eIF2Bε mutations in *gcn2*Δ strain KAY16 grown in SD medium (nonstarvation conditions). WT, wild type. (B) Growth of serially diluted cultures of the indicated eIF2Bε mutants in strain KAY16 on SD medium ± 3AT at the concentration indicated (left panels) and growth of a fixed cell concentration on SD medium containing a linear gradient of AZC. (C) Repeat of 3AT experiment from panel B, except that mutations were introduced on high-copy-number plasmids (H/C).

ing substrate concentration increased the rate of nucleotide release for eIF2B, as expected (compare open bars in Fig. 7B and C). However eIF2B$^{\beta V341D}$ activity was significantly lower than that in the wild type (solid bars) and was only modestly stimulated by increased substrate concentration. These assays demonstrate that eIF2B$^{\beta V341D}$ nucleotide exchange function is significantly compromised.

A closer inspection of the purified preparations used in this assay revealed there were imbalances in the Coomassie blue staining intensities of some subunits in our mutant complex. Specifically, when equal amounts of each preparation were analyzed (2.6 μg), levels of copurifying eIF2Bα, -β, and -δ subunits were reduced (compare Fig. 7A, lanes 3 and 6). Increasing the loading of the mutant complex to 4.0 μg (compare lanes 3 and 4) or reducing the loading of the wild-type complex to 1.7 μg (compare lanes 5 and 6) largely eliminated the eIF2Bα, -β, and -δ subunit imbalance, but instead introduced imbalances in the levels of eIF2Bγ and -ε. Thus it seemed likely that the activity of eIF2B$^{\beta V341D}$ was reduced at least in part because the complex was less stable. Our purification procedure was performed in buffer containing 500 mM KCl. We therefore decided to extend our analysis by performing immunoprecicipitation reactions at a lower, more physiological, salt concentration (150 mM). We made extracts from wild-type and eIF2Bβ mutant cells expressing eIF2B at normal levels and immunoprecipitated eIF2B with an eIF2Bε-specific antibody and probed each fraction with eIF2B and eIF2 subunit-specific antibodies. These experiments confirmed that there was no significant reduction in the overall expression levels of any subunit of eIF2B in the starting extracts (Fig. 7D, lanes 1 to 5). All four eIF2B subunits coprecipitated with eIF2Bε (lane 6). However, there were significant reductions in the levels of associated eIF2Bα, -β, and -δ subunits for eIF2Bβ^{V341D} (lane 9) and, to a lesser extent, also for eIF2Bβ^{G354V} (lane 10). Because eIF2Bε contains the major binding site for eIF2, it was not surprising that the amount of copurifying eIF2 was unaffected (Fig. 7D, bottom panel). These experiments imply that the V341D mutation destabilizes protein-protein interactions within the eIF2B complex sufficiently to reduce activity and hence alter both global translation initiation rates (lower) and gene-specific expression of *GCN4* (higher).

The main eIF2B structural defect observed in V341D mutant cells from the immunoprecipitated complexes is the dramatic exclusion of the eIF2Bδ subunit (Fig. 7D, lane 9). This result suggested that this mutation and to a lesser extent the adjacent G354V mutation may weaken the affinity between the normal δ subunit and one or more of the other subunits in the mutant eIF2B complex. If this was true, then increasing the concentration of the most excluded subunit may drive higher levels of complex formation. To test this idea, we transformed eIF2Bβ^{V341D} cells with a second plasmid overexpressing the

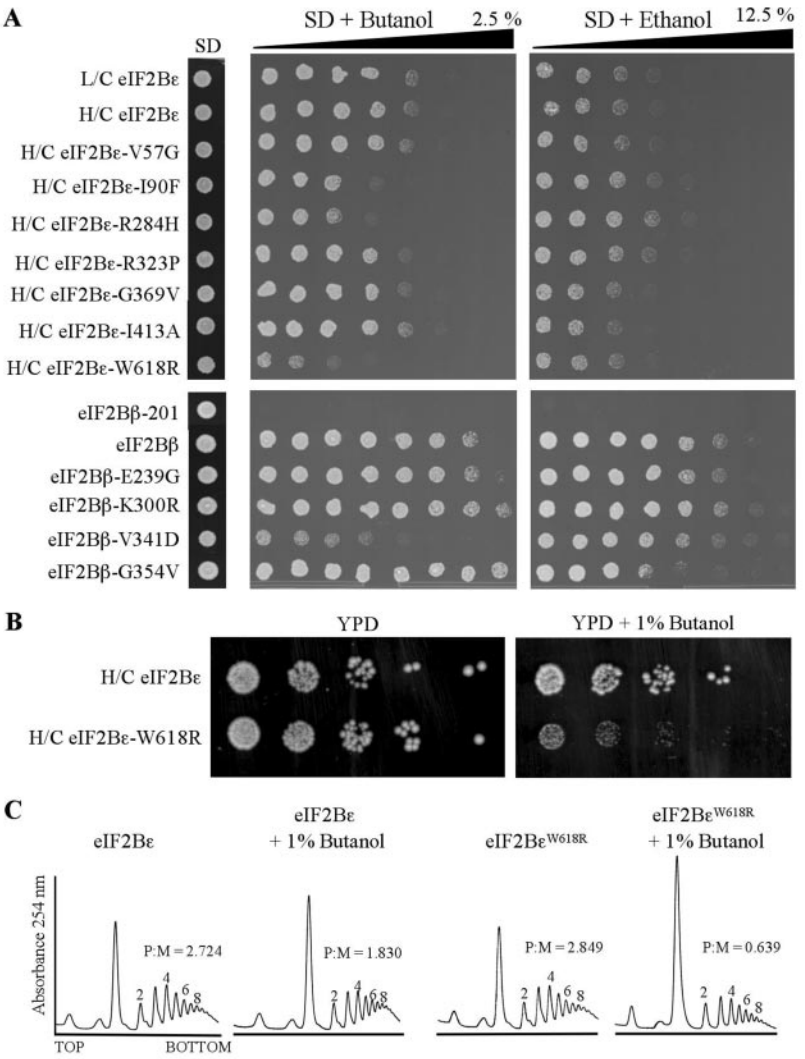

FIG. 6. Some CACH/VWM mutants display sensitivity to fusel alcohols. (A) The growth of eIF2Bε and eIF2Bβ CACH/VWM mutants and eIF2Bβ-201 was monitored on SD agar containing linear gradients of butanol or ethanol to maximum concentrations of 2.5 and 12.5%, respectively. (B) Fivefold serial dilution of high-copy-number eIF2Bε and eIF2Bε^{W618R} grown on YPD agar containing 1% (vol/vol) butanol. (C) Polysome profile analysis of high-copy-number eIF2Bε and eIF2Bε^{W618R} grown in YPD and following addition of 1% butanol for 10 min. P:M, polysome/monosome ratio.

native eIF2Bδ subunit alone and asked if it could rescue the rate of growth of this otherwise slow-growing strain. As controls, we included a vector-alone transformation and a plasmid expressing wild-type eIF2Bβ. In accord with our hypothesis, we found that the poor growth of these cells was rescued to a level identical to that seen when the cells were transformed with wild-type eIF2Bβ (Fig. 8A). In addition, high-copy-number eIF2Bδ could partly rescue the 3AT and AZC resistance phenotypes of eIF2Bβ^{V341D} cells (Fig. 8B and C). These results support the idea that the eIF2Bβ^{V341D} mutation reduces the affinity of eIF2Bδ for the complex in vivo rather than simply being an artifact observed only in cell extracts.

DISCUSSION

We set out to determine how mutations in eIF2B responsible for the human disorder CACH/VWM affect the function and regulation of eIF2B. We employed the well-characterized molecular/genetic system available in yeast so that we could rapidly assess a large number of mutations by a variety of molecular, genetic, and biochemical assays, assays not so readily available in other experimental systems. While acknowledging that yeast cells are evolutionarily far from humans, the conserved function and regulatory mechanisms shared between yeast and humans allowed us to directly test how these features of eIF2B may impact this disease. Importantly, an independent study has used transfection of human eIF2B gene cDNAs into a mammalian cell expression system to analyze several CACH/VWM mutations; that work has identified similar eIF2B defects to our study (34a).

CACH/VWM mutations reduce eIF2B activity. Previous studies have revealed that mutations in yeast eIF2B can act in at least three ways to alter its function. (i) They can reduce eIF2B function generally and may cause global changes in

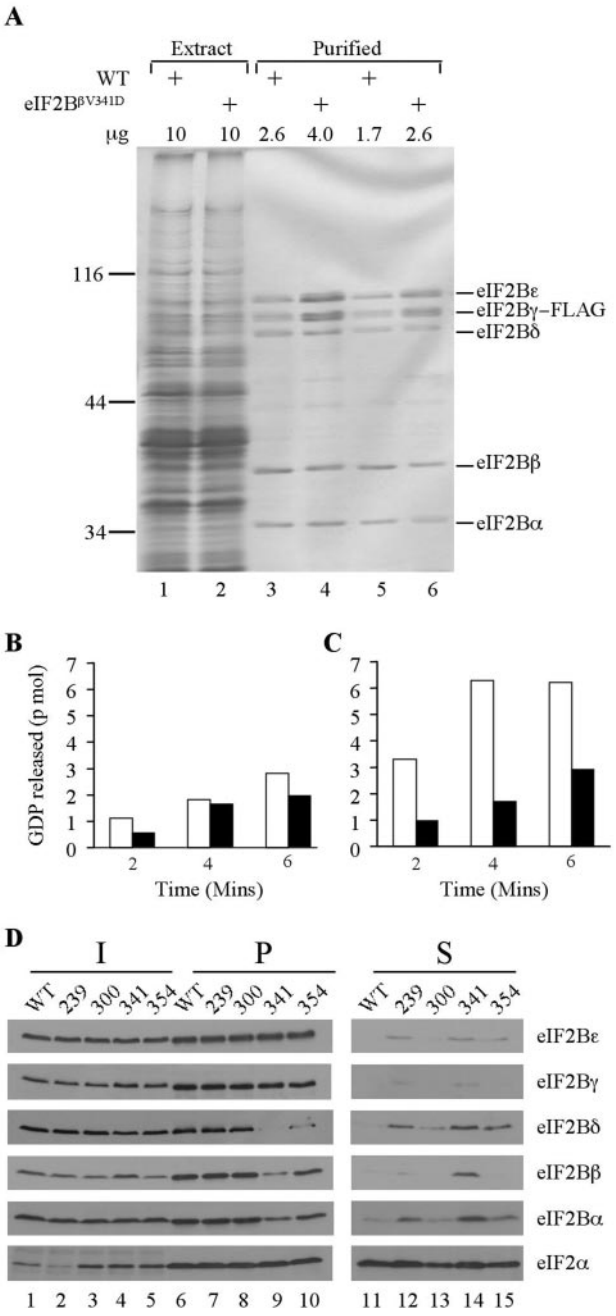

FIG. 7. eIF2Bβ^{V341D} reduces eIF2B nucleotide-exchange activity and destabilizes the eIF2B complex. (A) Coomassie-stained SDS-PAGE gel of FLAG-tagged eIF2B and eIF2B$^{\beta V341D}$ purified from strain BJ1995 transformed with high-copy-number plasmids overexpressing all five subunits of eIF2B. Loadings for each lane are given in micrograms. WT, wild type. (B and C) In vitro nucleotide exchange activity of FLAG-purified eIF2B (open bars) and eIF2B$^{\beta V341D}$ (solid bars) shown as picomoles of GDP released. Equal amounts (0.6 pmol) of wild-type eIF2B and eIF2B$^{\beta V341D}$ were used in each experiment with 15 pmol of eIF2-[^{3}H]GDP (B) or 23 pmol of eIF2-[^{3}H]GDP (C), respectively. (D) Coimmunoprecipitation of eIF2Bβ CACH/VWM mutant complexes. eIF2B was immunoprecipitated from extracts of cells from the wild type and the indicated eIF2Bβ mutants with anti-eIF2Bε antibody. Immunoblotting with the indicated polyclonal antiserum is shown. For each input fraction (I; lanes 1 to 5), 20 μg of total protein was loaded. Pellet fractions (P; lanes 6 to 10) contain the immunoprecipitated material from 125 μg of extract. For each supernatant fraction (S; lanes 11 to 15), 40 μg of unbound fraction was loaded in each lane.

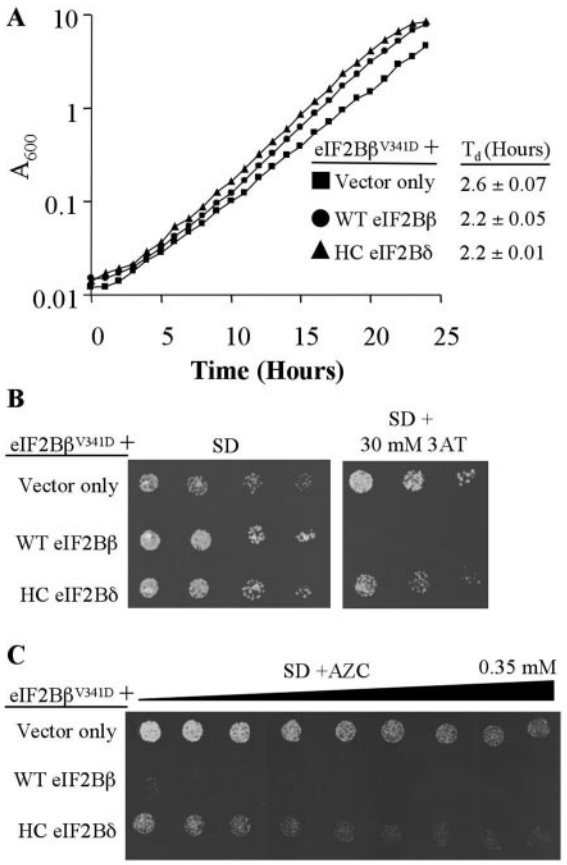

FIG. 8. Overexpression of eIF2Bδ rescues the growth defect of eIF2Bβ^{V341D} cells. (A) Growth of liquid SC-uracil cultures. The calculated doubling time (T_d) in liquid exponential-phase culture is indicated. Strains are *gcn2*Δ eIF2Bβ^{V341D} cells transformed with wild-type eIF2Bβ (●), high-copy-number (HC) eIF2Bδ (▲), or empty vector control (■). (B) Serial dilutions of the same strains on solid SD ± 3AT. (C) Growth of the same strains on an AZC gradient.

protein synthesis (*gcd*$^-$ mutations). (ii) Mutations may specifically alter responses to eIF2(αP) (*gcn*$^-$ mutations). (iii) Mutations can alter responses to butanol addition. Our analysis suggests that CACH/VWM mutations do not act by specifically failing to respond to eIF2(αP): they do not mimic yeast *gcn*$^-$ mutations. This is supported by the observation that in yeast, *gcn*$^-$ mutations have only been described in the eIF2Bα, -β, and -δ subunits (38) and within eIF2α (48). In contrast to this observation, CACH/VWM mutations have been found in all five eIF2B subunits, with the majority within eIF2Bε, while none have yet been found in eIF2α (47). Therefore, our analyses indicate that CACH/VWM mutations reduce the activity of eIF2B, not its downregulation by eIF2(αP).

Reduction in eIF2B function by CACH/VWM mutations alters translational controlled stress responses. CACH/VWM symptoms develop or progress after periods of head trauma and fever. This has prompted some to speculate that the eIF2B mutations responsible may be temperature sensitive. In yeast, some previously described eIF2B mutations are temperature-sensitive alleles (24). We therefore made extensive studies to assess if any mutations were temperature sensitive, with negative results (data not shown).

eIF2B is also regulated by a diverse array of stresses, many of which are known to activate one of the eIF2α kinases (see the introduction). It is not known which, if any, are important in oligodendrocytes, the cells mainly affected by CACH/VWM. However, in mammals, phosphorylation of eIF2 following head injury has been reported. Animal models show that damage caused by ischemia and reperfusion induces eIF2 phosphorylation in neurons, probably via activation of the kinase PERK (8). To assess the role of eIF2 phosphorylation on gene-specific translational control in our model system, we imposed amino acid starvation to monitor the expression of *GCN4*. Because of four short uORFs in the 5′ leader of its mRNA, Gcn4p levels are hypersensitive to reductions in eIF2B activity. *GCN4* expression inversely correlates with eIF2B activity. We found that many CACH/VWM mutations altered responses to amino acid starvation. *GCN4* translation was activated in the absence of Gcn2p activity, indicating reduced eIF2B function. Moderately affected alleles mutated in eIF2Bε revealed aberrant *GCN4* expression (Fig. 5), but had no impact on growth rate (Fig. 2) or general translation (polysome profiles not shown). The most severely affected allele we analyzed was eIF2Bβ^{V341D}, equivalent to *EIF2B2*-V316D in humans. This mutation dramatically elevated *GCN4* expression in the absence of amino acid starvation (Fig. 4) and caused a moderately severe impairment of growth rate (Fig. 2) and reduced rates of global translation, as evidenced by an elevated 80S peak during polysome analysis (Fig. 2C). We directly assessed the impact of the eIF2Bβ^{V341D} mutation on eIF2B activity in vitro and confirmed that activity was reduced significantly (Fig. 7).

We also analyzed the effects of CACH/VWM mutations on the recently described eIF2B-mediated response to butanol (3). We found that several mutations were impaired in butanol resistance, including one allele that was not altered in its response to amino acids (I90F, equivalent to human *EIF2B5*-L106F). Because the signaling pathway from butanol to eIF2B in yeast is not yet known, the significance of butanol sensitivity for CACH/VWM is unclear, but this phenotype remains a useful indicator of minor eIF2B defects.

Why do eIF2B mutations cause CACH/VWM? Two questions to consider here are: why eIF2B, as apposed to another translation factor? And why are oligodendrocytes the cells that are mainly affected? At this time, only speculation is possible. A relatively trivial explanation could be that in the affected cells, eIF2B expression levels may be low and limiting for translation. Any further reduction in activity caused by the eIF2B mutations would therefore prevent the cells from responding appropriately to trauma or infection, leading to the pathology observed. In favor of this idea, it was recently shown that eIF2B protein levels in the hippocampus of normal rats decrease with age (30). Alternatively, there may be one or more mRNAs whose translation is critical for function of the affected cells and whose expression is regulated via an eIF2B-dependent pathway. *GCN4*, as employed here, and *ATF4* are well-documented examples of such genes. Both of these genes are transcriptional activators that can directly or indirectly control levels of hundreds of genes (26, 36). It is not yet known if a similarly regulated gene is important in glial cells. Other hypomyelinating leukodystrophies that affect oligodendrocytes directly involve myelin protein mutations: e.g., myelin basic protein deficiency and Pelizaeus-Merzbacher disease (32). Myelin proteins are processed via the ER, so one possible contributor to CACH/VWM is ER stress activation of the eIF2α kinase PERK and subsequent down-regulation of eIF2B activity. There is only limited access to patient material at death, when the affected tissues are extremely damaged, making careful analysis difficult. Transgenic mouse models may be useful if aspects of pathology are preserved. However, normal mice already have one of the most common human CACH/VWM mutations yet found, so there is no guarantee of success. Our study may help choose which of the many human mutations identified to study in a mouse model system.

CACH/VWM mutations reduce the amount of five-subunit eIF2B complexes in cells. The CACH/VWM mutations that we studied in the α and ε subunits all reduced the steady-state expression level. For eIF2Bα^{N209Y}, this will form a mixture of four-subunit complexes (lacking α) and five-subunit complexes containing eIF2Bα^{N209Y}. In yeast, loss of eIF2Bα has no significant effect, except under amino acid starvation conditions. Accordingly, we found that yeast expressing this allele behaved exactly as a strain expressing no eIF2Bα subunit (data not shown). However, experiments using eIF2B purified from human (HeLa) cells have shown that loss of eIF2Bα does significantly reduce eIF2B activity, which can be restored following addition of recombinant eIF2Bα (51). So if human eIF2Bα^{N208Y} has similar effects on steady-state levels of eIF2Bα, as observed here, then cells would be expected to have both reduced eIF2B activity and impaired ability to respond to eIF2(αP). Similarly, all seven eIF2Bε alleles studied reduced eIF2Bε levels. Fewer active five-subunit complexes will form in these cells, causing reduced eIF2B activity. Because the amino acid changes affect single residues, one likely explanation for the reduced protein levels is that the tertiary structure of the affected subunit is aberrant. Misfolded proteins are often targeted for destruction (27). Under the growth conditions analyzed here, it appears that yeast has a greater eIF2B capacity than it requires; therefore, eliminating misfolded proteins will be preferential to forming aberrant eIF2B complexes. However, if aberrantly folded proteins are not eliminated from the cells affected by CACH/VWM, then presumably a greater number of defective eIF2B complexes will form.

The eIF2Bβ mutations studied did not affect the level of the protein expressed in cells, but consistent with altered folding, eIF2Bβ^{V341D} significantly reduced the stability of the five-subunit complex (Fig. 7). This indicates that this mutant forms an aberrant quaternary protein structure. The other three eIF2Bβ mutants appear to have only minor defects that resulted in small increases in the amounts of free subunits when compared with the wild type (Fig. 7D, lanes 11 to 15). The primary defect for the V341D mutation appears to be that the eIF2Bδ subunit affinity for the complex is reduced. Increasing the in vivo concentration of this subunit alone was sufficient to restore a normal growth rate and partly rescue amino acid biosynthesis defects (Fig. 8). The simplest interpretation of this result is that the missense mutation directly reduces the affinity of an interaction between the β and δ subunits that leads to exclusion of the δ subunit and hence impairs function.

Further support for the idea that CACH/VWM mutations alter eIF2B structural integrity comes from the recent X-ray crystal structure determination for the minimal catalytic do-

main of eIF2Bε (residues 544 to 704) (4a). Two residues altered in CACH/VWM are found within this fragment: human W628R and E650K. Yeast W618R is equivalent to W628R and was studied here. Both residues are buried within the interior of the determined structure, rather than being surface exposed. In agreement with our findings here, the amino acid substitutions are predicted to disrupt the structural integrity of this region rather than directly alter an interaction with eIF2. When combined, these results imply that CACH/VWM mutations act in more than one manner to achieve the same end result: lower eIF2B activity.

Aberrant protein folding is characteristic of several other human genetic disorders, including cystic fibrosis, where the most common mutation, CFTR-ΔF508, results in aberrant folding of the protein and enhanced degredation by the proteosome (18). In addition, several neurodegenerative disorders such as Huntington disease, and Alzheimer's and prion diseases are caused by accumulation of aggregated proteins with altered conformation. One therapeutic strategy being explored for cystic fibrosis is development of drugs that will improve the ability of the mutant proteins to correctly fold (40). This strategy may be one worth exploring for CACH/VWM.

ACKNOWLEDGMENTS

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