Characterization of the minimal catalytic domain within eIF2B: the guanine-nucleotide exchange factor for translation initiation

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For protein synthesis initiation in eukaryotes, eIF2B is the guanine-nucleotide exchange factor for eIF2. eIF2B is an essential multi-subunit factor and a major target for translational control in both yeast and mammalian cells. It was shown previously that the largest eIF2B subunit, eIF2Be, is the only single subunit with catalytic function. Here we report the results of a molecular dissection of the yeast e subunit encoded by GCD6 in which we have identified the catalytic domain. By analysis of a series of N-terminal deletions in vitro we find that the smallest catalytically active fragment contains residues 518–712 (termed Gcd6p518–712). Further deletion to position 581 (Gcd6p581–712) results in loss of nucleotide exchange function, but eIF2B-binding activity is retained. C-terminal deletion of only 61 residues (Gcd6p521–681) results in loss of both functions. Thus Gcd6p518–712 contains two regions that together constitute the catalytic domain of eIF2B. Finally, we show that the catalytic domain can provide eIF2B biological function in vivo when elevated levels eIF2 and tRNA Met are also present.

Keywords: eIF2/eIF2B/GCD6/guanine-nucleotide exchange/protein synthesis initiation

Introduction

Guanine-nucleotide binding (G) proteins control various key cellular processes. Many require accessory factors including nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that control their nucleotide-bound state. While G proteins themselves share common consensus nucleotide-binding motif sequences and structures within their nucleotide binding domains, the GEFs in particular are structurally and mechanistically diverse (Sprang and Coleman, 1998; Cherfils and Chardin, 1999), suggesting they do not arise from a common evolutionary ancestor.

eIF2 is a G protein composed of three subunits that is required for the initiation of protein synthesis in all eukaryotic cells. It forms a ternary complex with GTP and aminoacylated initiator methionyl tRNA (Met-tRNA Met) to deliver the latter to the 40S ribosomal subunit for each protein synthesis initiation event. Following mRNA selection and initiator codon recognition, eIF2-bound GTP is hydrolysed to release the Met-tRNA Met at the P site of mRNA bound 40S complexes by the GAP eIF5. eIF2-GDP is released from the ribosome and 60S subunit joining, promoted by a second G protein eIF5B, completes the initiation process. As eIF2 has a higher affinity for GDP than GTP it requires the action of a GEF to reform eIF2-GTP in order to participate in further translation initiation events. GEF function is provided by eIF2B (reviewed in Hinnebusch, 2000; Dever, 2002).

eIF2B is a large protein complex composed of five subunits, α to ε (smallest to largest). Missense mutations have recently been identified in all subunits of human eIF2B that are causal in the rare inherited and fatal neurodegenerative brain disorder leuкоencephalopathy with vanishing white matter (Leegwater et al., 2001; van der Knaap et al., 2002). This is the first genetic disorder known to affect translation factor genes directly.

eIF2B activity can be regulated in cells, and this has been shown to control both global and gene-specific translation initiation in yeast and mammalian cells. The most widely studied mechanism of eIF2B control involves phosphorylation of the α subunit of eIF2 in response to diverse cellular stresses by one of four protein kinases: HRI (or HCR) responds to haem deficiency in reticulocyte cells (Chen, 2000); PKR is part of the antiviral response and is activated by double-stranded RNAs (Kaufman, 2000); PERK (or PEK) responds to stress in the endoplasmic reticulum (Ron and Harding, 2000); and GCN2 responds to amino acid starvation (Hinnebusch, 2000). All four kinases phosphorylate eIF2 at a conserved serine at position 51 of the α subunit. This converts eIF2 from a substrate to a competitive inhibitor of eIF2B function. Reduction of eIF2B activity lowers ternary complex formation and can reduce global translation rates. In addition, translation of mRNAs that encode proteins required to respond to the cellular stress is elevated. The two best characterized examples of this are GCN4 in the yeast Saccharomyces cerevisiae and ATF4 in mammalian cells (Hinnebusch, 1996; Harding et al., 2000; Dever, 2002). In both cases, the presence of short open reading frames (ORFs) in the 5′ leaders of the mRNAs are required for translational control.

Previous genetic and biochemical experiments, mainly in yeast, have allowed progress in defining the roles for eIF2B subunits. The α, β and δ subunits share extensive sequence similarity with each other and can form a subcomplex in the absence of the other two subunits (Yang and Hinnebusch, 1996). Importantly, each plays a non-redundant role in sensing and binding eIF2α phosphorylated at Ser51, such that a single missense mutation in any one subunit can make eIF2B insensitive to the phosphorylation event (Vazquez de Aldana and Hinnebusch,
Results

N-terminal deletions of GCD6 identify a minimal fragment of elf2B that is active in vitro

Previous experiments with both yeast and mammalian elf2B (Fabian et al., 1997; Pavitt et al., 1998; Anthony et al., 2000) have shown that the ε subunit of elf2B (elf2Be) is the primary catalytic subunit of the GEF elf2B. Our analysis of missense and nonsense mutations in yeast elf2Be (Gcd6p) suggested that the C-terminal half of Gcd6p containing the catalytic domain (Gomez and Pavitt, 2000). To identify the minimal catalytically active region of Gcd6p, a series of N-terminal deletions of the GC56 ORF was created by PCR and inserted downstream of the GAL1 promoter in a 2 μm yeast expression plasmid to fuse the GC56 sequences to tandem N-terminal FLAG and His6 epitopes (FHε; see Supplementary data available at The EMBO Journal Online for details). Each deletion was designed to assess the role of a region of sequence conservation between GCD6 and its homologues from other eukaryotes, as shown schematically in Figure 1 (constructs 2–7). Previously, we showed that an in-frame internal deletion of residues 93–358 (Gcd6p<sup>93–358</sup>; construct 3 in Figure 1) retained in vitro nucleotide exchange and elf2 binding activity indistinguishable from the full-length isolated Gcd6p subunit (Gomez and Pavitt, 2000), so this construct was used as the starting point for the new series of deletions we made. As elf2 and elf2B co-purify at salt concentrations up to 500 mM, Gcd6p polypeptides were purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA resin in the presence of 1 M KCl to ensure that elf2 did not co-purify; this was confirmed by western blotting with antibodies specific for yeast elf2α (Gomez and Pavitt, 2000; data not shown). Immunoblot analysis with antibodies specific to the other four elf2B subunits confirmed that the N-terminally deleted Gcd6p proteins were purified free of contaminating elf2B subunits, while the purified full-length Gcd6p preparation contained low levels of full five-subunit elf2B (Figure 2A, compare lanes 5–8 with lanes 1 and 2; data not shown). These results confirm that the N-terminal half of elf2Be functions in elf2B complex formation.

Fig. 1. Summary of <em>in vivo</em> and <em>in vitro</em> results. Columns show: plasmid name; scheme of GC6 fragment expressed; N-terminal tag F (FLAG-His<sub>6</sub>) or GST; relative expression level in cells following growth in 4% galactose + 2% raffinose (SCGR) media as determined by western blotting with anti-FLAG M2 antibody or anti-GST antibody (scale 0–4); activity and elf2 binding <em>in vitro</em> of purified fragment indicated as + (yes), − (no) or nt (not tested); <em>in vivo</em> function in the presence of overexpressed elf2 and tRNA<sub>Met</sub> in strain GP41115 on agar containing 2% raffinose only (SCR, non-inducing conditions) or 4% galactose + 2% raffinose SCGR, (inducing conditions) where the rate of colony formation was assessed on a numerical scale 0–6, where ‘6’ is the rate of growth of wild-type GCD6 in this strain and ‘2’ is the rate of growth of the vector transformed strain on each medium.
Fig. 2. In vitro analysis of FH₄-tagged fragments of eIF2B (Gcd6p). (A) Western blot analysis of IMAC partially purified Gcd6p polypeptides using M2 anti-FLAG antibody (top panel) to detect Gcd6p and antisera specific for the other four eIF2B subunits to detect low levels of co-purifying proteins, as indicated to the right of each panel. Loadings were designed for 2-fold increments and for the top panel only are: lane 1, 75 ng; lanes 2 and 3, 150 ng; lane 4, 300 ng; lanes 5 and 7, 82.5 ng; lanes 6 and 8, 165 ng. For the lower four panels 33X the protein concentration loaded for the top panel was loaded in each lane (i.e. lane 1, 2.5 μg, etc.) The band corresponding to Gcn3p is indicated in the lower panel. (B) Western blot comparison of Gcd6p58-712 with full-length Gcd6p as in (A) using the M2 anti-FLAG antibody. Amount loaded in each lane corresponds to 1.5-fold increments: lanes 1 and 4, 89 ng; lanes 2 and 5, 133 ng; lanes 3 and 6, 200 ng. (C) Nucleotide exchange activity of purified proteins. Amount of [³H]GDP dissociated (pmol) from 7 pmol eIF2-GDP complexes following 3 min incubation with 2.5 μg partially purified Gcd6p or an equivalent amount of the indicated Gcd6p fragments [as determined from western analysis, (A) and (B), and data not shown] at 10°C in our standard filter-binding assay. Buffer indicates the eIF2B independent dissociation of [³H]GDP under these assay conditions. Mean errors are <5% for each assay. (D) Western blots showing binding of eIF2 (20 nM) to FLAG M2 affinity gel (lanes 2-8) pre-incubated with FH₄-Gcd6p proteins (lanes 3-8) or an equivalent concentration of FLAG peptide (200 nM, lane 2). Lane 1 (input) contains 6.25 ng of purified eIF2. Lanes 2-8 show the fraction of proteins remaining bound to resin after extensive washing, where 8.5% (top panel), 31% (middle panel) and 31% (bottom panel) of total bound material was loaded in each well. Membranes were probed with specific antisera, indicated to the right of each panel. Ig indicates the heavy and light chains of the precipitating FLAG M2 antibody.

Equivalent molar amounts of each protein were assayed for guanine-nucleotide exchange activity in a standard filter-binding assay where the loss of [³H]GDP from eIF2-³H]GDP binary complexes in the presence of a 100-fold excess of unlabelled GDP is assessed (Figure 2C). In these assays all N-terminal deletions assessed as active as the wild type, including the smallest fragment tested, Gcd6p518-712. In addition, we assessed the ability of these proteins to bind to eIF2. Gcd6p proteins (~200 nM) were incubated with anti-FLAG M2 affinity gel and then incubated with eIF2 (20 nM) in the presence of bovine serum albumin (BSA). The amount of eIF2 that remained bound to the resin after extensive washing was assessed by immunoblotting (Figure 2D). In accordance with the functional assay, all N-terminal deletions were active in this assay, including the smallest fragment tested, Gcd6p518-712.

Thus our smallest tested segment (at this point in our analysis), an ~200 amino acid segment, retained both eIF2 binding and nucleotide exchange activities. This segment contains two evolutionarily conserved regions of sequence similarity (Figure 3). The extreme C-terminal region (~60 residues) contains two elements rich in aromatic and acidic residues and is similar to a region in eIF5. This region has been termed the AA box or eIF5C homology region, and has been implicated in mediating binding to the eIF2B β subunit (Asano et al., 1999; Das and Maitra, 2000). The N-terminus (residues 518–593) is also conserved, but less is known about this region. We previously identified mutations altering residues 552 and 576, both of which impaired the nucleotide exchange activity (Gomez and Pavitt, 2000; asterisks in Figure 3). In addition, mammalian eIF2B activity can be regulated by changes in phosphorylation by GSK-3 in this region (Wang et al., 2001; SRAGS underlined in rat sequence in Figure 3), suggesting that this first conserved region may be important for eIF2B function. An alternative possibility was that this region only indirectly modulated eIF2B activity and that a minimal eIF2-binding fragment would suffice. To test these possibilities, we made a further deletion construct, Gcd6p581-712, using the same expression system. Unfortunately, we could not detect expression of this
Gcd6p fragment, suggesting that it was either not expressed efficiently or not stable once expressed (Figure 1, construct 7; data not shown).

To overcome the lack of expression of Gcd6p581–712, we reasoned that a longer N-terminal tag might stabilize the small protein. We therefore cloned the PCR product into an alternative galactose-inducible yeast expression plasmid that allowed the fusion the Gcd6p sequences to glutathione S-transferase (GST) from Schistosoma japonicum. As a positive control we also cloned the functional Gcd6p518–712 fragment into the same expression system. The expression of these constructs was assessed with anti-GST antibody and this demonstrated that both the resulting GST–Gcd6p518–712 and GST–Gcd6p581–712 proteins were efficiently expressed (Figure 1, constructs 10 and 11; data not shown). Proteins were purified using glutathione–Sepharose resin and eluted with reduced glutathione. Protein activities were assessed in both nucleotide exchange and in vitro binding assays, and identically purified GST alone was used as a negative control for all experiments (Figure 4). The nucleotide exchange assay clearly shows that this function is eliminated with the smaller Gcd6p581–712 fragment. As these new purified proteins lacked the FLAG epitope, the eIF2-binding assay was altered to employ glutathione–Sepharose resin. The results of this assay indicate that both GST–Gcd6p518–712 and GST–Gcd6p581–712 are able to bind to eIF2. While it is clear that the affinity of the smaller fragment is lower in this assay, it is significantly above the background binding observed for GST alone. Together these results suggest that two conserved regions of eIF2Be are critical for its catalytic function. The C-terminal region is sufficient for eIF2 binding alone, while the region from 518–580 enhances eIF2 binding and additionally provides nucleotide exchange activity. We have designated this evolutionarily conserved region the ‘catalytic centre’ in Figure 1.

**Deletion of the C-terminal 61 residues of Gcd6p eliminates in vitro function**

Previous studies have implicated the C-terminal ~60 residues of eIF2Be as important in binding eIF2 (Asano _et al._, 2004). However, the lack of these residues did not affect the binding properties of the GST–Gcd6p518–712 and GST–Gcd6p581–712 proteins, indicating that these residues may play a role in the eIF2 binding specificity. This is consistent with the notion that these residues may be required for specific interactions with eIF2 or other factors involved in the activation of eIF2. Overall, these results suggest that the C-terminal 61 residues of Gcd6p are not required for its catalytic activity.

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Fig. 3. Alignment of Gcd6p518–712 with the C-termini of eIF2Be sequences from other eukaryotes. eIF2Be sequences from *S. cerevisiae*, *Rattus norvegicus* (rat), *Drosophila melanogaster* and *Caenorhabditis elegans* were aligned using Clustal W. The region shown was selected and shaded with MacBoxshade v. 2.01. All identical residues are white type on black, three of four identical are shaded light grey, similar substitutions are shaded dark grey. The asterisks indicate previously mutated yeast residues. The GSK-3 phosphorylation site is underlined in the rat sequence (DDBJ/EMBL/GenBank accession numbers are, respectively, Z68195, Q64350, AL021086 and CAAF0163.1).

Fig. 4. In vitro analysis of GST-tagged fragments of eIF2Be (Gcd6p). (A) Nucleotide exchange activity of GST fusion proteins shown as rate of removal of [3H]GDP from eIF2 binary complexes. Four micrograms of each protein was used. GST (diamonds) GST–Gcd6p518–712 (squares) and GST–Gcd6p581–712 (circles). Error bars show standard deviations, trendlines are exponential curve fits to the data, broken line is for GST alone. (B) Interaction of eIF2 (20 nM) with GST fusion proteins (4 μg each, ~200 nM GST or ~100 nM Gcd6p518–712) using glutathione–Sepharose chromatography. Proteins indicated on the left were assessed by western blotting using the antiserum indicated on the right of each panel. Lanes 1–3, 0.25 μg input GST fusion proteins; lane 4, 1 μg input eIF2; lanes 5–10, upper panel, 10% of bound material loaded, lower panels, 55% of bound material loaded. The bottom panel is a longer exposure of the same experiment shown in the middle panel.
et al., 1999; Wang et al., 2001). However, our above results suggested that the region 518-580 also contributed to the eIF2 binding affinity. To assess the role of Gcd6p residues 652-712 in our in vitro assays, we made a construct where these residues were deleted (Gcd6p<sup>1-651</sup>, Figure 1, construct 8). This construct was expressed and we were able to purify it intact. In our yeast expression system, like all constructs containing an intact eIF2B<sub>ε</sub>N-terminus including the wild type (Gomez and Pavitt, 2000), Gcd6p<sup>1-651</sup> co-purified with low levels of the other four eIF2B subunits (Figure 2A). When nucleotide exchange activity was assessed, this construct was completely inactive; even when a 4-fold molar excess of protein was used [Figure 2C, Gcd6p<sup>1-651</sup> (4×)]. Our protein–protein interaction assay confirmed that specific eIF2 binding was eliminated with this construct (Figure 2D, lane 8). These results demonstrate the critical importance of residues 652-712 to eIF2B<sub>ε</sub> function.

We have shown previously that the eIF2β<sub>ε</sub>, β and δ subunits act together to sense and bind to eIF2 when the latter is phosphorylated on Ser51 of its α subunit by a regulatory kinase (see Introduction). Therefore, regions of the five-subunit eIF2B complex other than residues 618-712 of eIF2β<sub>ε</sub> may contribute to the binding surface with non-phosphorylated eIF2. It was therefore of interest to determine whether eIF2β<sub>ε</sub> residues 652-712 are as critical for five-subunit eIF2B function as they are in the isolated eIF2β<sub>ε</sub> subunit, or whether other determinants for eIF2 binding provided by other subunits can compensate. We therefore altered our protein expression system to purify five-subunit wild-type eIF2B and mutant eIF2B lacking Gcd6p residues 652-712 (eIF2B<sup>ε1-651</sup>). In this expression system, the eIF2B<sub>β</sub> subunit (Gcd1p) is FLAG- and His<sub>6</sub>-tagged at its C-terminus (see Materials and methods). Assays for nucleotide exchange activity and eIF2 binding showed that both functions were severely impaired (Figure 5). When the exchange activity of 2 μg of each protein was compared, the mutant activity was ~15% that of wild-type eIF2B in these assays. Increasing the protein concentration above 3 μg did not result in any enhanced rate of nucleotide exchange (data not shown), suggesting that one or more kinetic parameters for the mutant may be altered. As stable eIF2 binding was virtually eliminated in our assay we can conclude that residues 652-712 of Gcd6p provide the major eIF2-binding region.

**Gcd6p<sup>518-712</sup> provides eIF2B biological activity in vivo**

We wished to obtain in vivo results of biological activity to complement our in vitro biochemical studies. Four of the five genes encoding eIF2B subunits are essential in yeast (all but GCN3, encoding eIF2B<sub>α</sub>). As Gcd6p<sup>518-712</sup> and larger fragments are catalytically active in vitro, but possess reduced activity compared with five-subunit eIF2B, we reasoned that elevated expression of these fragments may provide eIF2B function in vivo. Initially we asked whether overexpression of catalytically active Gcd6p fragments from galactose-inducible expression plasmids were able to complement a deletion of GCD6 on galactose-containing medium. All results were negative (data not shown).

![Fig. 5. Impaired activities of eIF2B complex lacking the C-terminal 61 residues of the epsilon subunit. (A) Nucleotide exchange assay with indicated amounts of wild-type eIF2B (squares) eIF2B<sup>ε1-651</sup> (triangles, circles) or buffer control (diamonds). Trendlines are exponential curve fits to the data, broken line is for buffer control. (B) Interaction of eIF2 (5 nM) with five-subunit complexes of wild-type eIF2B (lane 2), eIF2B<sup>ε1-651</sup> (lane 3) or an equivalent amount of FLAG peptide (100 nM, lane 1) on anti-FLAG M2 resin. eIF2 and eIF2B subunits indicated on the left were probed with the specific antiserum indicated on the right, where 8.5% (eIF2B panels) and 31% (eIF2 panels) of total bound material was loaded in each lane.

Hannig and colleagues recently showed that they could delete the genes encoding all five subunits of eIF2B, providing they co-overexpressed all three subunits of eIF2 and one of the four genes encoding tRNA<sub>Met</sub> (Erickson et al., 2001). The resulting yeast strain was severely slow growing. We constructed a similar strain by transforming a gcdoΔ yeast strain bearing GCD6 on a low-copy plasmid with a multicopy plasmid co-overexpressing eIF2 and tRNA<sub>Met</sub> (the latter from the IMT<sub>4</sub> gene). Plasmid shuffling with 5-fluoro-orotic acid (5-FOA)-containing media was then used to select for cells that had segregated off the GCD6 plasmid. As expected from the previous report, all the resulting 5-FOA-resistant colonies were extremely slow-growing on rich media (Figure 6A) with a doubling time of ~6 h in exponential phase at 30°C (cf. 90 min for the parent strain, data not shown). Three independent 5-FOA-resistant, slow-growing colonies were assessed by immunoblotting for the presence of Gcd6p. All showed that Gcd6p was no longer present, confirming that the GCD6 plasmid had been lost from these cells.
Fig. 6. Minimal eIF2B catalytic domain provides nucleotide exchange activity in vivo. (A) Growth of strains GP4113 (left) and GP4115 (right) on YPD agar. Photographed after 5 days at 30°C. (B) Western blot analysis of GP4113 (lane 1) and three 5-FOA-resistant colonies (lanes 2–4), with GP4115 in lane 2. Fifteen micrograms of total soluble cell protein was loaded in each lane. Blot was probed with the specific antibodies indicated to the right of each panel. (C) Growth of indicated transformants of strain GP4115 on SCGR minus uracil, leucine, isoleucine and valine (SCGR-ULV) media. ‘Vector’ indicates plasmid pRS316 (Siesski and Hieter, 1989) and ‘GCD6‘ the low copy GCD6 URA3 plasmid pB85 (Bushman et al., 1993); other plasmids are indicated in Figure 1. Times listed are doubling times ± SD (n) for each strain in liquid SCGR-ULV during exponential growth phase. (D) Growth of indicated transformants of strain GP4181 on SCGR-uracil media. ‘GCD2‘ indicates the low copy GCD2 URA3 plasmid pCP62 (Pavitt et al., 1997).

(Figure 6B, lanes 2–4). Thus, in our strain only the catalytic subunit of eIF2B is deleted, but this strain has a growth rate similar to the Hann laboratory strain where all five eIF2B subunits were deleted. One strain (called GP4115) was used for further analysis.

We reasoned that in this strain the overexpressed eIF2 and tRNA\textsuperscript{Met} provided barely sufficient ternary complex formation rates in the absence of eIF2B function for cell growth; therefore, if catalytically active Gcd6p fragments were also expressed in these cells the rate of ternary complex formation and hence growth would be enhanced. Accordingly, strain GP4115 was transformed with plasmids to express different fragments of Gcd6p and growth rates were assessed on media containing 2% raffinose media, where the GAL1 promoter is not induced and on the same media containing 4% galactose (Figures 1, columns SCR and SCGR, respectively, and 6C). In complete agreement with the in vitro analysis, the results show that all Gcd6p that were catalytically active in our in vitro biochemical assays improve the rate of growth of yeast in vivo. The smallest biologically active Gcd6p fragment in this in vivo assay is Gcd6p\textsuperscript{518-712}. When the growth rates were compared quantitatively by monitoring \( A\text{max} \) in liquid SCGR media, both control vectors and the non-catalytic Gcd6p\textsuperscript{581-712} fragment expressing cells grew with a doubling time of ~360–370 min. This was improved to ~250 min in cells expressing the Gcd6p\textsuperscript{518-712} catalytic fragment and to ~190 min for cells expressing native eIF2B (Figure 6C; data not shown).

In the analysis described above, we asked for complementation of gcd6\textsuperscript{Δ} only. It was therefore possible that the rescue of growth rate we observed in this assay was either due to the action of the overexpressed Gcd6p polypeptide alone, or was due to complex formation of the expressed Gcd6p fragments with the other four eIF2B subunits still present in this strain. From our previous analyses (see Figure 2A; Gomez and Pavitt, 2000) and that of others (Anthony et al., 2000), we believe that an intact eIF2Be N-terminus is required for eIF2B complex formation. This would suggest that Gcd6p\textsuperscript{518-712} acts independently of the other four eIF2B subunits present in this strain to promote eIF2B biological activity. To provide an additional test of this interpretation, we repeated the growth rescue assay using a strain deleted for GCD2, which encodes the eIF2Be subunit. In a gcd2\textsuperscript{Δ} strain transformed with Gcd6p fragments, no five-subunit eIF2B can form, and therefore any growth rescue must be due to the overexpressed Gcd6p fragments acting alone. This new strain (GP4181) was made in an identical manner to GP4115 and the series of transformation experiments was repeated with comparable results (Figure 6D; data not shown). Together, these data demonstrate that Gcd6p\textsuperscript{518-712} comprises the minimal catalytically and biologically active domain of eIF2B.

Discussion

In this study, we used a combination of in vitro functional assays and in vivo growth assays to determine the minimal fragment of eIF2B capable of catalysing nucleotide exchange. We find that a small ~200 residue, ~23 kDa region at the C-terminus of the largest subunit, eIF2Be (Gcd6p in yeast), contains the sequences necessary for...
both eIF2 binding and exchange of guanine nucleotides. The γ subunit of eIF2B shares extensive sequence similarity over its entire length with the N-terminal 467 residues of eIF2Bγ (Figure 1). These conserved regions appear to be required for protein–protein interactions between eIF2B subunits and are not required for minimal catalytic function of eIF2Bγ (Figure 2; Gómez and Pavitt, 2000). The level of activity observed for this fragment was indistinguishable from the activity of the full-length isolated Gcd6p subunit (Figure 2; data not shown). This represents a reduction of at least 10-fold in the rate of exchange when compared in similar assays with the full five-subunit factor (Gómez and Pavitt, 2000). It was therefore not surprising that this fragment could not support the growth of a deletion of GCD6 without some additional help. Remarkably, Hannig and co-workers observed that cells with excess eIF2 and tRNA<sup>Met</sup> are able to survive without eIF2B function, but have a severe growth defect (Erickson et al., 2001; Figure 6). This suggested that the rate of eIF2B-independent eIF2-GTP-Met-tRNA<sup>Met</sup> ternary complex formation in these strains is severely growth limiting. We have shown here that expression of the Gcd6p<sup>518–712</sup> catalytic fragment greatly enhanced the rate of growth of strains deleted for eIF2B subunits that are normally essential proteins. Together these findings provide strong evidence that Gcd6p<sup>518–712</sup> is the minimal catalytic segment of eIF2B.

The C-terminal –60 residues provide the major domain for interaction with non-phosphorylated eIF2

When the sequence of Gcd6p<sup>518–712</sup> is compared with the equivalent region from other eukaryotes, two obvious regions of relatively high sequence similarity are evident (top and bottom row in Figure 3), which are separated by a poorly conserved region. The sequences of the extreme C-terminal region of eIF2B are conserved with eIF5, which binds eIF2-GTP-Met-tRNA<sup>Met</sup> to activate GTP hydrolysis during translation initiation. Asano et al. (1999) found that mutation of seven or 12 conserved acidic or aromatic residues within this region of yeast eIF2B to alanines (termed AA boxes 1 and 2) reduced binding of eIF2B to eIF2β. Similar results were reported for equivalent mutations in eIF5. Wang et al. (2001) provided evidence that this region was also important in mammalian eIF2B. Mutation of two serine residues (S706 and S707 in the rat sequence), both substrates for casein kinase II phosphorylation, reduced both eIF2 interaction and activity in vitro. While these studies implicated this region of eIF2B in eIF2 binding it was possible that the missense mutations altered the folding of this region of the molecule and that this indirectly weakens eIF2–eIF2B interaction. However, our results demonstrate that this region alone is sufficient for eIF2 binding activity (Figure 4B; construct GST–Gcd6p<sup>581–712</sup>). We also found that deletion of these residues in construct Gcd6p<sup>1–651</sup> eliminates both stable eIF2 binding and nucleotide exchange activity of the isolated eIF2Bγ subunit (Figure 2), demonstrating that this region is both necessary and sufficient for binding eIF2. When the purified mutant five-subunit complex (eIF2B<sup>1–651</sup>) was analysed, we found a substantial reduction in function in both assays (Figure 5). Therefore, in contrast to the results with the single subunit where eIF2 binding and GDP exchange functions were completely eliminated, some residual activity remained. These findings suggest that the other four eIF2B subunits can provide some eIF2-binding activity, at least in vitro.

Residues 518–580 contain the eIF2B catalytic centre

The region from residues 518–580 of yeast eIF2Bγ is essential for its catalytic activity, and enhances eIF2 binding (Figure 4). This region is also conserved in other eIF2B sequences (Figure 3, top row). These results strongly suggest that residues required for catalysis are within this region of eIF2B. We previously identified missense mutations within this region at T552 and S576 that reduced the catalytic activity of Gcd6p and led to a slow-growth phenotype in vivo (Gómez and Pavitt, 2000). These findings are consistent with this region being important for eIF2B function and suggest that this conserved region contains catalytic residues of eIF2B. We have therefore termed this region the ‘catalytic centre’. It is interesting that phosphorylation of mammalian eIF2B by GSK-3 in this region (the rat sequence is underlined in Figure 3) can reduce mammalian eIF2B activity in a similar way to the yeast mutations at T552 and S576. We suggest that all these modifications to the structure in this region directly impair the catalytic function of eIF2B.

Residues within the catalytic centre region that are conserved through all eIF2B proteins are good candidates to be directly involved in catalysis. It is noteworthy that residues with charged and/or bulky hydrophobic side chains have been implicated in catalysis of nucleotide exchange for other GEF proteins where G protein-GEF co-crystal structures have been determined. Several of the conserved eIF2Bγ residues have similar side chains. However, subsequent mutational analysis has not always borne out these predictions. For example, in translation elongation factors *Escherichia coli* EF-Ts in complex with EF-Tu, EF-Ts residues D80 and F81 in the conserved TDFV motif were implicated in mediating nucleotide exchange (Kawashima et al., 1996). However, mutation of these two residues affected EF-Ts function only 2- to 3-fold each, or 10-fold when both were altered (Zhang et al., 1996). Instead, mutations altering EF-Ts interaction with EF-Tu had a more pronounced effect on rates of nucleotide exchange (Zhang et al., 1998; Wieden et al., 2002). In contrast to this, K205 of *S.cerevisiae* eEF1Bγ in its complex with eEF1A has a catalytic role (Andersen et al., 2001). Therefore, in conclusion, directed mutagenesis of conserved residues within the catalytic centre region may provide clues to which residues of eIF2B are directly involved in catalysis of nucleotide exchange and future work will address this.

Models for action of the minimal catalytic domain

The results presented and discussed here are consistent with a two-step binding model for guanine-nucleotide release catalysed by eIF2B. In this model (shown in Figure 7A) the C-terminal AA box domain of eIF2Bγ makes contact with the lysine-rich domain of eIF2Bβ (Asano et al., 1999). This interaction then allows binding of the catalytic centre region and nucleotide exchange to proceed. Where the catalytic centre interacts with eIF2 is
not known. As all known GEFs bind directly to the G protein subunit to mediate nucleotide exchange, this seems a likely option for eIF2. In contrast, Gcd6p<sup>581–712</sup> can only make the AA box–eIF2β interaction (Figure 7B). However, as no direct interaction between eIF2B and eIF2γ has been detected to date, it is possible that another mechanism may operate. Perhaps binding of the eIF2B catalytic centre to eIF2β induces a conformational change that is transmitted from eIF2β to eIF2γ to induce nucleotide exchange (Figure 7C).

Models very similar to these for eIF2B function could apply to eIF5 action. eIF5 stimulates GTP hydrolysis within 40S ribosome-bound eIF2-GTP-Met-tRNA<sub>Met</sub> ternary complexes. eIF5 shares a similar C-terminal AA box eIF2β-binding domain with eIF2Bε, and it was recently demonstrated that a separate conserved N-terminal domain is required for eIF5 to stimulate GTPase activity (Das et al., 2001; Paulin et al., 2001). Therefore, the arrangement of similar C-terminal eIF2β binding domains (AA boxes) and separate catalytic domains that promote GEF and GAP function appears to be common to these eIF2-controlling translation factors. However, eIF2B functions with eIF2-GDP while eIF5 acts on eIF2-GTP-Met-tRNA<sub>Met</sub>; therefore, although the AA box–eIF2β interaction is necessary for both proteins to interact with eIF2, these interactions alone are unlikely to determine the specificity of binding. For eIF2B this specificity may be due to the catalytic centre. Our results suggest that the remainder of the ~294 kDa five-subunit eIF2B complex functions to stimulate and regulate this activity to control the rate of translation, either globally or for specific mRNAs.

**Materials and methods**

**Yeast cell culture**

Standard yeast media were used throughout (Rose et al., 1990); YPD (2% yeast extract, 1% peptone, 2% dextrose); SC (synthetic complete with 2% dextrose); SCB (synthetic complete with 4% galactose and 2% raffinose); SCGR (synthetic complete with 4% galactose and 2% raffinose). Amino acids and/or uracil were omitted from SC media to maintain plasmid selection as necessary.

**Yeast strains and genetic methods**

Standard genetic methods were used to construct yeast strains (Guthrie and Fink, 1991). Transformation of yeast strains with plasmids was by the lithium acetate method (Gietz et al., 1995). Plasmid shuffling employing 5-FOA was carried out as described previously (Boeke et al., 1987). Yeast strains used are described in Table I. PEP4 was disrupted in GP3667 using a pep4::LEU2 allele as described previously (Pavitt et al., 1998) to create GP3889. Deletion of PEP4 enhanced expression levels and reduced proteolysis of many of the Gcd6p fragments made (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Construction/reference</th>
</tr>
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<tbody>
<tr>
<td>GP3040</td>
<td>MATa ura3-52 trp1-63 leu2-3 ura3-2 leu2-112 gcl2Δ pCP62[URA3 CEN4 GCD2]</td>
<td>Pavitt et al. (1997)</td>
</tr>
<tr>
<td>GP3511</td>
<td>MATa leu2-3 leu2-112 ura3-52::his4-lacZ ino1 gcn2Δ pep4::LEU2 sui2Δ pAV1089[SU2 SU3 GCD11-6×His 2 μm URA3]</td>
<td>Pavitt et al. (1998)</td>
</tr>
<tr>
<td>KAY16/GP3750</td>
<td>MATa ura3-52 trp1-63 leu2-3 ura3-2 leu2-112 gcl2Δ pBB85[GD6 CEN6 URA3]</td>
<td>Asano et al. (1999)</td>
</tr>
<tr>
<td>GP3667</td>
<td>MATα trp1-63 ura3-52 leu2-3 leu2-112 GAL2 gcn2Δ</td>
<td>Gomez and Pavitt (2000)</td>
</tr>
<tr>
<td>GP3889</td>
<td>MATα trp1-63 ura3-52 leu2-3 leu2-112 GAL2 gcn2Δ pep4::LEU2</td>
<td>pep4::LEU2 in GP3667; this study</td>
</tr>
<tr>
<td>GP4113</td>
<td>KAY16' + pAV1732[h.c. eIF2 IMT4 2 μm LEU2]</td>
<td>Transformation of KAY16; this study</td>
</tr>
<tr>
<td>GP4115</td>
<td>MATa ura3-52 trp1-63 leu2-3 leu2-112 gcl2Δ pAV1732[SU2 SU3 GCD11-6×His IMT4 2 μm LEU2]</td>
<td>GP4113 following 5-FOA growth; this study</td>
</tr>
<tr>
<td>GP4173</td>
<td>MATa ura3-52 trp1-63 leu2-3 leu2-112 gcl2Δ pCP62[URA3 CEN4 GCD2]</td>
<td>Transformation of GP4040; this study</td>
</tr>
<tr>
<td>GP4181</td>
<td>MATa ura3-52 trp1-63 leu2-3 leu2-112 gcl2Δ pAV1732[h.c. eIF2 IMT4 LEU2]</td>
<td>GP4173 following 5-FOA growth; this study</td>
</tr>
</tbody>
</table>
Plasmid constructions
Standard methods were used to construct all plasmids (Sambrook and Russell, 2001). Details can be found in the Supplementary data.

Protein expression and purification from yeast
His-tagged yeast elf2 was purified from strain GP3511 by immobilized metal affinity chromatography (IMAC) using Ni-NTA resin (Qiagen) followed by heparin–Sepharose chromatography (Amersham) as described previously (Pavitt et al., 1998). As strain GP3511 is deleted for the sole yeast elf2zr kinase (gen zA), elf2 preparations are entirely non-phosphorylated at the regulatory Ser51 site. Expression of FHc-tagged Gcd6p fragments in strains GP3667 or GP3889 and their purification by IMAC using Ni-NTA resin was as described previously (Gomez and Pavitt, 2000). Purification of FHc-tagged wild-type five-subunit elf2B overexpressed in strain GP3866 was also by IMAC using Ni-NTA resin as described previously (Gomez and Pavitt, 2000). Purification of FHc five-subunit elf2B(1-145) was identical to that for wild type except that pAV1687 (2 μM URA3 FHc-GCD1 g687pS525R) was transformed into strain GP3889 in place of pAV1533 (2 μM URA3 FHc-GCD1 GCD6p) with pAV1494 (2 μM LEU2 GNC3 GCD7 GDC2).

GST fusion protein expression and purification
Plasmids for GST and GST fusion protein expression were transformed into GP3889 and SC in geuracil, leucine, isoleucine and valine (SG-ULIV) to saturation. Cells from these cultures were subcultured into SGAs-ULIV (0.45% w/v glucose + 2% w/v galactose) and grown to A600 3–5. All subsequent steps were performed at 0–4°C. Cells were harvested by centrifugation at 5000 g for 15 min, washed and resuspended in 2 ml/g wet wt ice-cold GST lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM MgCl2, 1 mM diithiothreitol, 1× complete EDTA-free protease inhibitor tablet (Roche)) and cells were lysed by vortexing with glass beads. The resulting cell lysate was clarified by sequential centrifugation at 5000 g for 5 min and 100 000 g for 1 h. Supernatants were applied to a Hi trap GST 1 ml column, pre-equilibrated with the same buffer. After extensive washing, the bound proteins were eluted with reduced glutathione (Sigma) in elution buffer (100 mM Tris–HCl pH 8.8, 120 mM KCl, 0.1% Triton, 20 mM reduced glutathione). Protein-containing fractions were combined, dialysed using (10 kDa mol. wt cut off) dialysis cassettes (Pierce) into storage buffer (100 mM Tris–HCl pH 8.0, 100 mM KCl, 3 mM MgCl2, 5% glycerol, 5 mM β-mercaptoethanol) and stored in aliquots at –80°C. Protein concentrations were determined by the Bradford assay (Bio-Rad) using BSA as a standard (Bradford, 1976).

In vitro nucleotide exchange assays
In vitro nucleotide exchange assays using elf2-1HGDGP substrate and 100-fold excess unlabelled GDP and partially purified elf2B or elf2Be (Gcd6p) fragments were performed as described previously (Pavitt et al., 1998; Gomez and Pavitt, 2000).

In vitro protein–protein interaction assays
Assays for interaction between elf2 and FHc-tagged Gcd6p proteins were performed using FLA1 M2 affinity resin as described previously (Gomez and Pavitt, 2000). Assays where GST-tagged Gcd6p fragments replaced FHc-tagged Gcd6p were performed similarly, but using glutathione-Sepharose 4B resin (Amersham Bioscience) in place of FLAG M2 resin. Purified GST or GST–Gcd6p fusion proteins (~4 μg) were bound to prewashed glutathione–Sepharose resin (40 μl of 50% slurry) made up to 400 μl with binding buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 2 mM MgCl2, 5 mM β-mercaptoethanol containing 2.5 mg/ml BSA) at 4°C for 2 h on a blood tube rotator. Resin was pelleted by low-speed centrifugation (2000 g for 2 min) and washed three times to remove any unbound protein, and 1 μg elf2 was added with fresh binding buffer. Incubation was continued for 2 h and the washing steps were repeated. Proteins remaining bound to the resin were eluted into 20 μl 1× Laemmli sample buffer (Sambrook and Russell, 2001).

Immunoblotting
Proteins and protein:protein interaction assays were analysed by SDS–PAGE on 12% PAGE gels. Proteins were visualized by immunoblotting using specific antisera: mouse monoclonal αFLAG M2 (Sigma); rabbit polyclonal αGST (Sigma); elf2B and elf2 subunit-specific rabbit polyclonal antisera have been described previously (Dever et al., 1995). Detection was by horseradish peroxidase-linked anti-rabbit/mouse secondary antibodies and enhanced chemiluminescence (Amersham Biosciences).

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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References


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