Cloning and expression of cDNAs for the β subunit of eukaryotic initiation factor-2B, the guanine nucleotide exchange factor for eukaryotic initiation factor-2

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A key control point in the initiation of protein synthesis in mammalian cells is the recycling of eukaryotic initiation factor (eIF)-2 by the guanine nucleotide exchange factor eIF-2B. In mammalian cells, eIF-2B is a complex of five different subunits termed ϵ , δ , γ , β and α . To clone cDNAs for the β subunit of rabbit eIF-2B, amino acid sequence data was first obtained and used to design redundant oligonucleotide primers for use in PCR. PCR products were used to screen a rabbit liver cDNA library in λ gt11 to obtain full-length cDNAs for eIF-2B β . The cDNAs were sequenced completely on both strands and revealed an open reading frame encoding a predicted 351-amino acid

polypeptide of 39.0 kDa. The molecular mass and pI (5.99) of the predicted protein agree well with the properties of eIF-2B β purified from rabbit reticulocytes. *In vitro* transcription/translation of the cDNAs gave rise to a product that migrated at a position indistinguishable from that of this subunit of the purified protein. The amino acid sequence shows a high degree of similarity to that of GCD7, a *Saccharomyces cerevisiae* protein thought to be equivalent to mammalian eIF-2B β . Northern-blot analysis revealed a single major mRNA species for eIF-2B β in each of the four rabbit tissues tested.

INTRODUCTION

In the initiation of protein synthesis in mammalian cells, the initiator tRNA (Met-tRNA_i^{Met}) is carried to the 40 S ribosomal subunit as part of a ternary complex with eukaryotic initiation factor (eIF)-2 and GTP. Later in the initiation process, the GTP is hydrolysed and the eIF-2 is released in a binary complex with GDP. In this form it is inactive and must be recycled to its GTP-bound form before it can participate in a further round of initiation. This recycling requires a second initiation factor, the guanine nucleotide exchange factor, eIF-2B (reviewed in [1,2]).

Mammalian eIF-2B is a complex of five distinct subunits [3–13] termed ϵ , δ , γ , β and α [14]. In this respect, it differs from other guanine nucleotide exchange factors that are normally single polypeptide chains. A complex of five subunits (GCD6, GCD2, GCD1, GCD7 and GCN3) thought to be equivalent to mammalian eIF-2B has recently been identified in the yeast *Saccharomyces cerevisiae* [15]. All five of these proteins are involved in the translational control of the transcription factor GCN4 [16]. The suggestion that the GCD (general control derepressing) complex represents yeast eIF-2B is supported by sequence similarities between the yeast proteins and sequences available for subunits of mammalian eIF-2B. For example, the GCD6 sequence aligns well with that of eIF-2B ϵ [17] and the GCD2 sequence with that of eIF-2B δ [18]. cDNAs are available for all of the subunits of the yeast complex [17,19–21] but for mammalian eIF-2B, only the sequences of the ϵ [17] and δ [18] subunits have so far been reported.

The complexity of eIF-2B, relative to other exchange factors, raises the possibility that it might have roles other than guanine nucleotide exchange. Alternatively, the extra complexity could reflect the manner of its regulation. The recycling of eIF-2 catalysed by eIF-2B represents an important control point in

translation initiation and the activity of eIF-2B can be regulated in a number of different ways. NADP⁺/NADPH, NAD⁺/NADH and polyamines, such as spermine and spermidine, are allosteric effectors [4]; however, the physiological significance of these regulatory mechanisms is unclear. Mammalian eIF-2B can be phosphorylated on the largest of its subunits, the ϵ , by casein kinases-1 and -2 [4,22] and also by the insulin-inactivated kinase GSK-3 [23]. Phosphorylation by these kinases may well play an important role in the control of eIF-2B activity and hence in the control of protein synthesis initiation but, as yet, this is not well understood.

The best-understood mechanism for the control of eIF-2B activity is through phosphorylation of its substrate, eIF-2. When eIF-2 is phosphorylated on its α subunit, it acts as a potent competitive inhibitor of eIF-2B [24]. Inhibition of eIF-2B means that eIF-2.GDP is not recycled and initiation soon ceases. The level of eIF-2 phosphorylation has been shown to increase under certain conditions where protein synthesis is inhibited. These include physiological stresses such as heat shock (reviewed in [25]).

Three eIF-2 α kinases have so far been characterized: HRI (haemin regulated inhibitor) [26,27], PKR (double-stranded RNA-activated protein kinase) [28] and GCN2 [29]. GCN2 is a *S. cerevisiae* protein that plays an essential role in the translational regulation of GCN4 under conditions of amino acid starvation. GCN2 has been shown to be an eIF-2 α kinase and it is assumed that the phosphorylated eIF-2 inhibits yeast eIF-2B, leading, via an unusual mechanism, to the up-regulation of GCN4 [29]. Genetic studies have suggested that up to three subunits of the GCD complex in *S. cerevisiae* (GCN3, GCD2 and GCD7) may be involved in the interaction between the complex and eIF-2 phosphorylated in the α site (eIF-2 α P) that leads to inhibition of guanine nucleotide exchange [30]. By analogy, the genetic

Abbreviations used: HRI, haemin regulated inhibitor; PKR, double-stranded RNA-activated protein kinase; GCD, general control derepressing; GCN, general control non-derepressing; eIF, eukaryotic initiation factor; EF, elongation factor; eEF, eukaryotic elongation factor.

The nucleotide sequence of rabbit eIF-2B β appears in the EMBL database under the accession number Z48222.

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evidence from the yeast system could suggest possible roles for some of the subunits of mammalian eIF-2B.

Amino acid starvation also leads to a decrease in the rate of protein synthesis in mammalian cells. Since eIF-2 α phosphorylation is elevated under these conditions, it is assumed that translation is inhibited via decreased activity of eIF-2B (reviewed in [31]). It will be interesting to see if there exists a mammalian kinase equivalent to GCN2.

We have been attempting to clone cDNAs encoding subunits of mammalian eIF-2B as a first step in increasing our understanding of the roles of the individual subunits in the complex. In this paper we report the cloning of cDNAs for the β subunit of rabbit eIF-2B. The β subunit is potentially interesting for a number of reasons. First, the yeast equivalent of the β subunit, GCD7, may be involved in the interaction of eIF-2B with eIF-2 α P that leads to inhibition of guanine nucleotide exchange [32]. Secondly, Dholakia et al. have provided evidence for a binding site for GTP on this subunit [33].

EXPERIMENTAL

Materials

All chemicals and biochemicals were from Sigma Chemical Co. or BDH except where otherwise stated. Restriction endonucleases, T4 polynucleotide kinase, *Taq* polymerase and herring sperm DNA were from Boehringer-Mannheim, Lewes, E. Sussex, U.K. T4 DNA ligase, deoxyribonucleic acids, *Sma*I-cut, dephosphorylated pUC18 DNA and Ficoll (type 400) were from Pharmacia, Milton Keynes, U.K. Random 14-mer DNA labelling kit, [α -³²P]dCTP and [α -³⁵S]dATP were from DuPont-NEN, Stevenage, Herts., U.K. The oligo(dT)-primed rabbit liver λ gt11 cDNA library was from Clontech, Palo Alto, CA, U.S.A. Riboclon cDNA synthesis system (AMV reverse transcriptase, oligo(dT)₁₅ primer), polyATtract mRNA isolation system and TNT T7/T3-coupled reticulocyte lysate system were from Promega, Southampton, U.K. Problott membrane was from Applied Biosystems, Warrington, U.K. Nitrocellulose Extra Blotting Membrane was from Sartorius AG, Göttingen, Germany. Sequenase version 2.0 DNA sequencing kit was from United States Biochemicals. 'Hyperfilm' X-ray film and Hybond-N membrane were from Amersham International.

N-terminal sequencing of eIF-2B β

Subunits of purified rabbit reticulocyte eIF-2B [4] were resolved by SDS/PAGE and transferred to Problott membrane. N-terminal sequence of the β subunit was obtained by automated Edman degradation using an Applied Biosystems 477A sequencer.

Sequencing of peptides derived from eIF-2B β

Bands containing purified rabbit reticulocyte eIF-2B β were excised from Coomassie Blue-stained SDS/polyacrylamide gels. CNBr digestion was carried out in the gel chips in 70% formic acid and the resulting peptides were separated by tricine gel electrophoresis [34] and transferred to Problott membrane. N-terminal sequences of the CNBr-cleaved fragments were obtained as above.

Design of redundant oligonucleotide primers

The N-terminal protein sequence and the peptide sequences were used in the design of redundant oligonucleotide primers for use in the PCR. In total, six primers were produced with redundancies

ranging from 54- to 432-fold: A, CC(T/C/G)GG(T/C/G)-GC(G/C/T)AC(G/C/T)GAGAA(G/A)GG [sense, encoding PGATEK(G)]; B, AT(T/C/A)AT(T/C/A)(C/A)G(T/C/G)-GAGGAGTA(T/C)GG [sense, encoding IIREEY(G)]; C, TT(C/T)GT(G/T/C)GAGGAC(G/T/C)(T/C)T(G/C/T)AA(G/A)(A/C)G [sense, encoding FVEALK(R)]; D, CCGTA(C/T)-TC(C/T)TC(G/C/T)C(G/T)(G/T)AT(T/G/A)AT [antisense, encoding (G)YEERII]; E, TC(C/T)TC(C/T)TC(G/A)TT(G/T/C)GG(A/G)AA(C/T)TG [antisense, encoding (D)EENPFQI]; and F, CC(T/G)AT(T/G)AT(C/T/G)AC(C/T)TT(G/A)TT(C/T/G/A)AC [antisense, encoding (G)IIVKNV].

The orientation of the primer pairs was based on alignment of the eIF-2B β peptide sequences with the protein sequence of GCD7 [17], the yeast equivalent. These alignments were also used to predict the sizes of PCR products expected for each combination of primers.

PCR amplification of rabbit eIF-2B β cDNA

Rabbit liver total RNA was used for oligo(dT)-primed cDNA synthesis catalysed by AMV reverse transcriptase. The cDNA was then used as the template in PCR reactions.

Probe labelling

PCR products for use as probes for library screening, were labelled with [α -³²P]dCTP using a random-priming kit from DuPont-NEN.

Library screening

Bacteriophage DNA was transferred to Nitrocellulose Extra Blotting Membrane (Sartorius) and hybridized at 42 °C for 16 h in a solution containing 50% (v/v) formamide/5 $\times$ SSPE/2.5 $\times$ Denhardts/0.2 mg/ml sheared herring sperm DNA/0.1% (w/v) SDS. [1 $\times$ SSPE is 0.5 mM EDTA/0.15 M NaCl/10 mM NaH₂PO₄, H₂O; 1 $\times$ Denhardts is 0.1% (w/v) BSA (fraction V)/0.1% (w/v) Ficoll (type 400)/0.1% (w/v) polyvinylpyrrolidone]. Clone purification required secondary and tertiary screening under the same hybridization conditions.

Purification of bacteriophage DNA

Bacteriophage DNA was prepared from a 200 ml culture using the protocol outlined in the Clontech library screening manual.

PCR amplification of the 3' and 5' ends of the insert

In order to check if the cloned eIF-2B β cDNA was full-length, non-redundant primers for eIF-2B β (G and H) were used in combination with λ gt11 vector primers to amplify the 3' and 5' ends of the cloned cDNA. The purified bacteriophage DNA was used as template in the PCR reactions. The PCR products were cloned into pUC18 and subjected to DNA sequence analysis.

PCR amplification of the complete eIF-2B β cDNA

Non-redundant oligonucleotide primers (I and J) based on the 3' and 5' ends of the λ gt11 cDNA insert were used to PCR amplify complete eIF-2B β cDNAs using purified bacteriophage DNA as a template.

DNA sequence analysis

Sequencing was carried out on both strands using a Sequenase version 2.0 DNA sequencing kit with [α -³⁵S]dATP and using a

custom primer walking strategy. Sequence alignments were carried out using the LASERGENE suite of programs (DNASTAR, West Ealing, London, U.K.).

In vitro expression of eIF-2B β cDNAs

All three eIF-2B β cDNAs were subcloned into pBluescript (Stratagene) for *in vitro* expression in the reticulocyte lysate system. Expression was carried out using the T7/T3-coupled reticulocyte lysate system from Promega. Products were analysed by SDS/PAGE and autoradiography.

Analysis of eIF-2B β mRNA by Northern blotting

Total RNA was prepared from rabbit reticulocytes, liver, kidney and spleen using TRIzol reagent (BRL). For each tissue, 0.5 mg of total RNA was used in the production of poly(A)⁺ RNA using the polyATtract mRNA isolation system from Promega. The poly(A)⁺ RNA was separated on a formaldehyde gel and transferred to Hybond-N membrane. The membrane was probed using the 798 bp eIF-2B β PCR product labelled using a random-priming protocol as previously described. A phosphorimager was used to visualize the Northern blot and then the membrane was stripped and reprobed with a probe for actin labelled as described previously. The result was again obtained using the phosphorimager.

RESULTS

Cloning of cDNAs encoding the β subunit of rabbit eIF-2B

N-terminal protein sequence and peptide sequences for rabbit reticulocyte eIF-2B β were used in the design of redundant

oligonucleotide primers for use in the PCR. Alignment of eIF-2B β peptide sequences with yeast GCD7 suggested the correct relative orientation of primer pairs and allowed for prediction of the size of PCR product expected from each combination of primers.

All possible combinations of the redundant oligonucleotide primers were used in the PCR to attempt to amplify the rabbit liver eIF-2B β cDNA. PCR products were cloned into pUC18 and subjected to DNA sequence analysis. Three products were obtained that showed a high degree of similarity to GCD7 and contained regions of eIF-2B β sequence previously obtained by peptide sequencing. The longest PCR fragment (the product of primers C and E, see Figure 1) was 798 bp in length covering approx. 75% of the predicted coding region. The two other products were contained within this sequence.

The 798 bp PCR product was used to screen a rabbit liver cDNA library in λ gt11. Approx. 10⁶ bacteriophage plaques were screened and a single positive was obtained. Secondary and tertiary screens were used to purify the clone. A small amount of bacteriophage DNA was purified and PCR was used to amplify the 3' and 5' ends of the λ gt11 insert. These products showed the eIF-2B β cDNA coding region to be complete.

Non-redundant PCR primers designed to the 3' and 5' ends of the λ gt11 insert were used to PCR-amplify full-length rabbit eIF-2B β cDNAs using the purified bacteriophage DNA as template. Two separate PCR reactions were carried out. The products were cloned into pUC18 and three clones were selected for complete sequencing on both strands. The three sequences obtained differed from each other at one or two nucleotide positions (due presumably to PCR errors) but were otherwise identical and the wild-type rabbit eIF-2B β sequence was readily obtained.

This sequence is given in Figure 1 along with the deduced amino acid sequence. Figure 1 also shows 5' and 3' non-coding

Figure 1 Nucleotide and deduced amino acid sequences of rabbit eIF-2B β cDNA, from a λ gt11 library

Underlined regions of the protein sequence denote the sequences of CNBr-cleaved peptides obtained from rabbit eIF-2B β and the N-terminal protein sequence data. The positions of the redundant oligonucleotide primers A-F used to obtain the initial PCR products and the primers G-J used for additional PCR amplifications are shown.

Poly(A)⁺ RNA was prepared from rabbit reticulocytes, liver, kidney and spleen, and subjected to Northern-blot analysis. The membrane was probed for eIF-2B β . In order to try to establish the relative amounts of mRNA loaded from the different tissues, the Northern blot was stripped and reprobed for actin (a widely applied normalization procedure). The result is shown in Figure

Figure 4 Northern-blot analysis of eIF-2B β mRNA

Poly(A)⁺ RNA was subjected to Northern-blot analysis as described in the Experimental section. Phosphorimager printouts of a Northern blot probed for eIF-2B β and actin. The arrow on the left indicates the position of a weak band of higher molecular mass in the eIF-2B β panel. The positions of the 28 S and 18 S ribosomal RNAs are indicated.

4. The relative intensity of the eIF-2B β signal, compared with that for actin, is the same for each tissue, suggesting that the amount of eIF-2B β mRNA is approximately the same in each tissue. There is a single main band for eIF-2B β in each tissue, indicating the presence of a single major mRNA species. Closer inspection of the Northern blot reveals the presence of weak higher-molecular-mass bands for eIF-2B β . Such bands could represent additional mRNA species, or may be due to the presence of partially processed transcripts.

DISCUSSION

We report here the cloning of cDNAs encoding the β subunit of the rabbit guanine nucleotide exchange factor, eIF-2B. A consensus sequence for the coding region was obtained from three separate PCR products. The sequence has an open reading frame of 1053 nucleotides encoding a predicted 351-amino-acid polypeptide. The predicted molecular mass of 39.0 kDa is in agreement with the molecular mass of the β subunit estimated from SDS/PAGE gels of eIF-2B purified from rabbit reticulocytes [4] and when translated *in vitro*, all three clones give rise to products that migrate identically to the β subunit of purified eIF-2B. The deduced amino acid sequence has a predicted pI of 5.99 which corresponds very well with the value of around 6 estimated from isoelectric focusing gels.

The deduced amino acid sequence of the rabbit eIF-2B β has a high degree of similarity to that of the yeast GCD7 protein. In the yeast system it has been suggested that GCD7 plays a regulatory rather than a catalytic role in the eIF-2B complex [32]. The high degree of sequence similarity observed between GCD7 and rabbit eIF-2B β suggests that the mammalian subunit may play a similar role. Vazquez de Aldana and Hinnebusch report that certain mutations in GCD7 appear to make eIF-2B insensitive to inhibition via phosphorylation of eIF-2 α [32]. Although it depends on the exact alignment used, the residues mentioned do not seem to be rigidly conserved between GCD7 and the rabbit eIF-2B β sequence reported here. However, two of these residues are conservatively replaced in the rabbit β -sequence: aspartate 178 in GCD7 aligns with glutamate in rabbit eIF-2B β and isoleucine 348 in GCD7 aligns with leucine in the mammalian sequence.

The sequence of eIF-2B β also shows similarity to the C-terminus of eIF-2B δ [18]. This is similar to the situation for the

corresponding yeast polypeptides, where there is considerable identity between the sequence of GCD7 and the C-terminal sequence of GCD2 [30].

The rabbit eIF-2B β sequence has no obvious consensus motifs. In particular, it does not contain a consensus GTP-binding motif. This is surprising in view of the evidence of Dholakia *et al.* who reported that eIF-2B binds GTP and that GTP analogues can be cross-linked to the β subunit [33].

The presence or absence of a binding site for GTP on eIF-2B has important mechanistic implications. Two main models have been suggested to explain the mechanism of guanine nucleotide exchange by eIF-2B. In the substituted enzyme model, eIF-2.GDP binds to eIF-2B leading to the dissociation of the GDP. GTP then binds to eIF-2 and the eIF-2.GTP dissociates from eIF-2B. This model is supported by certain kinetic data [24] and also represents the mechanism of other guanine nucleotide exchange factors such as the bacterial protein elongation factor (EF)-Ts [36] and its mammalian counterpart eukaryotic elongation factor (eEF)-1 $\beta\gamma\delta$ [37,38].

The ternary complex model involves the formation of a complex containing eIF-2B and both substrates; eIF-2.GDP and GTP. The main feature of this model is that it requires a binding site for GTP on eIF-2B. This model is supported by evidence that purified eIF-2B cannot in itself promote the release of GDP from eIF-2 [4,39], free guanine nucleotides also being required, suggesting a mechanistic role for GTP. It is also consistent with the reported binding site for GTP on eIF-2B [33].

It is thus of interest that none of the subunits of yeast eIF-2B possesses a consensus GTP-binding motif. Since we report here that the sequence of rabbit eIF-2B β also lacks this motif, it seems unlikely that eIF-2B catalyses guanine nucleotide exchange via the ternary complex mechanism. There remains, however, the possibility that GTP could be binding to a non-consensus site.

The eIF-2B β sequence contains potential phosphorylation sites for several protein kinases. However, there is no evidence that this subunit is phosphorylated *in vivo* and it is not phosphorylated by a range of protein kinases that have been tested *in vitro* (B. L. Craddock, N. T. Price and C. G. Proud, unpublished work).

Northern-blot analysis reveals the presence of a single main species of mRNA for eIF-2B β in the four tissues tested. This is in agreement with results from Western blotting which show a single band for the β subunit of purified eIF-2B [35] and thus does not indicate the presence of multiple isoforms of this subunit. In contrast, a recent report by Henderson *et al.* predicts the presence of more than one isoform for the δ subunit of eIF-2B in mouse [40].

We now aim to overexpress eIF-2B β alone and in combination with other subunits of eIF-2B. This should enable us to conduct experiments which may help to elucidate the function of this subunit, its mechanism of action and the polypeptides and ligands with which it interacts.

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