Regulation of Guanine Nucleotide Exchange through Phosphorylation of Eukaryotic Initiation Factor eIF2α

ROLE OF THE α- AND δ-SUBUNITS OF eIF2B

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The guanine nucleotide exchange activity of eIF2B plays a key regulatory role in the translation initiation phase of protein synthesis. The activity is markedly inhibited when the substrate, i.e. eIF2, is phosphorylated on Ser51 of its α-subunit. Genetic studies in yeast implicate the α-, β-, and δ-subunits of eIF2B in mediating the inhibition by substrate phosphorylation. However, the mechanism involved in the inhibition has not been defined biochemically. In the present study, we have co-expressed the five subunits of rat eIF2B in Sf9 cells using the baculovirus system and have purified the recombinant holoprotein to >90% homogeneity. We have also expressed and purified a four-subunit eIF2B complex lacking the α-subunit. Both the five- and four-subunit forms of eIF2B exhibit similar rates of guanine nucleotide exchange activity using unphosphorylated eIF2 as substrate. The five-subunit form is inhibited by preincubation with phosphorylated eIF2 (eIF2(αP)) and exhibits little exchange activity when eIF2(αP) is used as substrate. In contrast, eIF2B lacking the α-subunit is insensitive to inhibition by eIF2(αP) and is able to exchange guanine nucleotide using eIF2(αP) as substrate at a faster rate compared with the five-subunit eIF2B. Finally, a double point mutation in the δ-subunit of eIF2B has been identified that results in insensitivity to inhibition by eIF2(αP) and exhibits little exchange activity when eIF2(αP) is used as substrate. The results provide the first direct biochemical evidence that the α- and δ-subunits of eIF2B are involved in mediating the effect of substrate phosphorylation.

Regulation of translation initiation plays an important role in the control of gene expression in eukaryotic cells (reviewed in Ref. 1). By modulating different steps in the initiation pathway, regulation of the translation of mRNAs coding for specific classes of proteins as well as regulation of overall mRNA translation can be achieved. The initiation pathway is composed of a number of discrete steps involving at least 12 unique proteins referred to as eukaryotic initiation factors, or eIFs. Of the many steps in translation initiation, only two are thought to be important in regulating the process in vivo. The two steps include the sequential binding of first mRNA and then initiator methionyl-tRNA (met-tRNAi) to the 40 S ribosomal subunit. In the latter step, eIF2 binds to the 40 S ribosomal subunit as a ternary complex with GTP and met-tRNAi. With formation of the 80 S initiation complex, the GTP is hydrolyzed and eIF2 is released as an eIF2-GDP binary complex. The GDP bound to eIF2 must then be exchanged for GTP, a reaction catalyzed by a guanine nucleotide exchange protein, termed eIF2B (reviewed in Ref. 2). In contrast to most guanine nucleotide exchange proteins, which are usually small and consist of a single subunit, eIF2B is composed of five dissimilar subunits present in equimolar amounts in a heteropentameric complex. Although eIF2B has been available in purified form from mammalian cells for 15 years (3), no information is available about the role of the individual subunits in catalyzing the guanine nucleotide exchange reaction. Furthermore, little is known about the role of the individual subunits in mediating regulation of the exchange activity.

The best characterized means of regulating eIF2B activity involves phosphorylation of the α-subunit of its substrate, eIF2, on Ser51. Phosphorylation of eIF2α converts eIF2 from a substrate into a competitive inhibitor (reviewed in Ref. 1). Based on genetic studies in Saccharomyces cerevisiae, it has been concluded that the eIF2α α-, β-, and δ-subunits are important in mediating the effect of substrate inhibition of eIF2B (4, 5). In S. cerevisiae deprived of amino acids, eIF2α becomes phosphorylated, leading to increased translation of a protein termed GCN4 (reviewed in Refs. 6 and 7). Although not established experimentally, it has been assumed that eIF2B activity in yeast is inhibited in response to eIF2α phosphorylation and that the inhibition of eIF2B activity is responsible for the increased translation of GCN4 mRNAs. This assumption is based on the finding that deletion of the α-subunit or point mutations identified in the α-, β-, and δ-subunits of eIF2B prevent the increase in translation of GCN4 mRNA in response to amino acid deprivation (4) without having any effect on cellular growth in nonstarved cells. In addition, overexpression of the eIF2B α-, β-, and δ-subunits leads to formation of a stable eIF2B subcomplex that overcomes the inhibitory effect of high level eIF2α phosphorylation, presumably through a mechanism involving sequestration of the phosphorylated eIF2 by the eIF2B subcomplex (5). The biochemical basis for the apparent insensitivity to eIF2α phosphorylation in cells expressing mutant forms of eIF2B subunits is unknown.

In the present study, the five subunits of rat eIF2B were co-expressed in Sf9 cells using the baculovirus expression system. A functional, five-subunit eIF2B complex was purified...
from the cells. In addition, a four-subunit complex lacking the α-subunit was expressed and purified. Both the four- and five-subunit forms of eIF2B exhibited similar specific activities, indicating that the α-subunit of the protein is not required for optimal guanine nucleotide exchange activity. However, whereas the four-subunit form of eIF2B was not inhibited by eIF2α(p), the five subunit form was. Furthermore, the exchange activity using eIF2α(p) as substrate was greater for four- than five-subunit eIF2B. Finally, the δ-subunit containing a double point mutation corresponding to mutations identified in yeast was expressed in combination with the other four subunits of eIF2B. The sensitivity of eIF2B containing the mutant δ-subunit to inhibition by eIF2α(p) was similar to that observed for eIF2B lacking the α-subunit. However, unlike eIF2B lacking the α-subunit, the ability of eIF2B containing the mutant δ-subunit to catalyze GDP exchange using eIF2α(p) was the same as wild-type eIF2B. Overall, the results provide the first biochemical evidence of the regulatory role of the α- and δ-subunits in mediating inhibition of exchange activity by substrate phosphorylation. In addition, they show that the α-subunit of eIF2B is not required for exchange activity.

MATERIALS AND METHODS

Expression of the Five Subunits of Rat eIF2B in Sf9 Cells—High titer stocks of baculoviruses encoding the wild-type eIF2B α, β, β′, and δ subunits were generated as described previously (8). For coexpression of eIF2B subunits, 2 × 10⁸ Sf9 insect cells were infected for 1 h in a reduced volume of 15 ml containing each of the three different virus stocks at a multiplicity of infection of 2–5 for each virus. The infected Sf9 cells were then transferred to a 250-ml Erlenmeyer flask containing 85 ml of SF-900 serum-free medium (Life Technologies, Inc.) and were maintained in culture at 28 °C using an orbital shaker (100 rpm). At 72 h after infection, 1-ml aliquots were removed and centrifuged in Eppendorf tubes at 2000 rpm for 3 min and pellets were stored at −70 °C until lysis.

Construction of the E311K,L315Q Mutant Form of eIF2Bδ—Site-directed mutagenesis (Altered Sites mutagenesis kit, Promega) was utilized to introduce specific changes to the eIF2Bδ cDNA. Oligonucleotide GPAD140 (5′-ATT TGC CTG AGA TGA CTG ACC AAT CTT TTG CAC ACA TGG-3′ was used to change Glu to Lys and Gln, respectively (eIF2B-E311K,L315Q), generating pAV1057. Plasmid pAV1153 (eIF2Bδ-E311K,L315Q) was created by subcloning the mutated eIF2Bδ 590-base pair BamHI-NdeI fragments from pAV1057 into identically cleaved J203 (also called pAC-2BaFLAG/G3FLAG). Nucleotide sequencing of the subcloned 590-base pair fragment confirmed that the plasmid contained only the expected site-directed nucleotide substitutions (underlined in the above oligonucleotide sequence).

Purification of Recombinant eIF2B from Sf9 Cells—The proteins expressed in Sf9 cells were immunopurified by chromatography on a matrix containing an immobilized anti-FLAG monoclonal antibody (Affi-Gel 1000 Affinity Gel; Bio-Rad). Briefly, the cells were lysed as described previously (8), and the lysate was centrifuged at 10,000 g at 4 °C. The supernatant was mixed with 2 ml of affinity matrix containing an immobilized anti-FLAG monoclonal antibody (Anti-FLAG M2 Affinity Gel; IBI/Kodak). Briefly, the cells were lysed as described previously (8), and the lysate was centrifuged at 10,000 g at 4 °C. The supernatant was mixed with 2 ml of affinity matrix containing an immobilized anti-FLAG monoclonal antibody (Anti-FLAG M2 Affinity Gel; IBI/Kodak). The column was washed with 30 ml of buffer B, followed by 30 ml of buffer C (20 mM Tris, pH 8.0, 150 mM NaCl), and the bound protein was eluted with 200 ml of buffer D containing 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.5% Triton X-100. The sample was then concentrated using a Millipore Biomax 50K centrifugal concentrator and stored in aliquots at −70 °C.

RESULTS

The five rat eIF2B subunits were coexpressed in Sf9 cells using the baculovirus expression system as described previously (8). Each of the recombinant proteins was expressed with an amino-terminal octapeptide extension referred to as FLAG to aid in purification. As shown in the left panel of Fig. 1, a single immunoaffinity purification step utilizing an anti-FLAG monoclonal antibody coupled to a solid matrix resulted in isolation of an approximately equimolar mixture of the five eIF2B subunits at a purity of greater than 90%. Likewise, expression of just the eIF2B β-, γ-, δ-, and ϵ-subunits yielded a complex lacking the α-subunit (Fig. 1, right panel). The four-subunit complex lacking the α-subunit will be hereafter referred to as eIF2Bδ(−α).

The specific activities of eIF2B and eIF2Bδ(−α) were compared in a guanine nucleotide exchange assay using eIF2α[H]GDP as substrate. As shown in Fig. 2, exchange activities were the same when equal amounts of eIF2B and eIF2Bδ(−α) were added to the assay. The results show that the α-subunit of eIF2B is not required for exchange activity.

In a previous study, incubation of Sf9 cell extracts expressing rat eIF2B with eIF2 phosphorylated with the eIF2α kinase, HCR, resulted in a decrease in exchange activity compared with extracts incubated with unphosphorylated eIF2 (8). In contrast, extracts of cells expressing eIF2βδ(−α) showed no decrease in exchange activity when incubated with eIF2 phosphorilated on the α-subunit. In the present study, purified eIF2Bδ and eIF2Bδ(−α) were incubated with either unphosphorylated eIF2 or eIF2α(p) prior to assay. As shown in the inset to Fig. 3 (lane 1), there was no detectable unphosphorylated eIF2 in the phosphorylated eIF2 preparation. Similarly, no phosphorylated eIF2δ was detected in the unphosphorylated preparation (Fig. 3, lane 2). In confirmation of the results obtained previously with crude cell extracts, incubation of purified eIF2B with eIF2α(p) (Fig. 3, open symbols) prior to assay resulted in a substantial decrease in exchange activity compared with incubation with unphosphorylated eIF2 (Fig. 3, closed symbols). In contrast, the activity of eIF2Bδ(−α) was nearly unaffected by phosphorylated eIF2.

The sensitivity of the exchange activity of eIF2B and eIF2Bδ(−α) to eIF2 phosphorylation was further examined using an eIF2α(p)[H]GDP complex as substrate. As shown in the top panel of Fig. 4, both eIF2B and eIF2Bδ(−α) catalyzed GDP exchange using phosphorylated eIF2, although the exchange activity using eIF2α(p) as substrate (open symbols) was
of eIF2 were then added to a reaction mixture containing 0.5 mM HCR; lane 2, HCR; lane 3, eIF2 phosphorylated with HCR; lane 4, eIF2B. The activity of eIF2 was measured as the exchange of [3H]GDP bound to eIF2 for nonradioabeled GDP with time as described under "Materials and Methods." The results represent the mean ± S.E. of three experiments. Within each experiment, two independent assays were performed for each condition.

The phosphorylated and unphosphorylated forms of eIF2 was formed as described previously (9). Thirty-five minutes after adding ATP and HCR by chromatography on a phosphocellulose column (24), the phosphorylated and unphosphorylated eIF2 was incubated for 2 min at 37 °C with 1.4 μg of either four-subunit or five-subunit eIF2B. The activity of eIF2B was measured as the exchange of [3H]GDP bound to eIF2 for free nonradiolabeled GDP with time as described under "Materials and Methods." The results represent the mean ± S.E. of three experiments. Within each experiment, two independent assays were performed for each condition.

The phosphorylated and unphosphorylated forms of eIF2 were then used as substrate for either four-subunit or five-subunit eIF2B. In addition, the exchange of [3H]GDP bound to eIF2 for free nonradiolabeled GDP was measured in the absence of eIF2B. The results represent the mean ± S.E. of three experiments. Within each experiment, two independent assays were performed for each condition. Bottom panel, an aliquot of the eIF2B reaction mixture was analyzed for eIF2 phosphorylation state prior to the start of the assay (t = 0) and at the end of the assay (t = 6 min). The reaction mixture was resolved by SDS-polyacrylamide gel electrophoresis, followed by protein immunoblot analysis using an antibody specific for eIF2 phosphorylated on Ser51.

The five-subunit eIF2B holoprotein, but not eIF2B lacking the α-subunit, is inhibited by phosphorylated eIF2. eIF2 was phosphorylated using the eIF2α kinase, HCR, and then separated from ATP and HCR by chromatography on a phosphocellulose column (24). The phosphorylated and unphosphorylated forms of eIF2α were resolved by slab gel isoelectric focusing and then electrophoretically transferred to a polyvinylidene difluoride membrane. The proteins were then visualized by protein immunoblot analysis using a monoclonal anti-eIF2α antibody (inset). Lane 1, eIF2 incubated in the absence of HCR; lane 2, eIF2 phosphorylated with HCR; lane 3, an extract from a rat liver perfused with histidinol showing resolution of a mixture of phosphorylated and unphosphorylated eIF2α. Approximately 0.5 μg of the five-subunit or four-subunit forms of eIF2α were incubated for 2 min at 37 °C with 1 μg of either unphosphorylated eIF2α or eIF2 phosphorylated on the α-subunit. The guanine nucleotide exchange activity of eIF2B was then measured as described in the legend to Fig. 2 using a binary complex of unphosphorylated eIF2α and [3H]GDP. The results represent the mean ± S.E. of three experiments. Within each experiment, two independent assays were performed for each condition.

Within the present study, the same double mutation was made in rat eIF2α (P) as substrate at a significantly faster rate than did five subunit eIF2B. The exchange activity observed was not influenced by substrate dephosphorylation since the amount of eIF2α(P) was the same at the beginning and end of the assay (Fig. 4, bottom panel). Likewise, the difference in exchange activity between the four- and five-subunit forms of eIF2B was not due to substrate dephosphorylation.

Pavitt et al. (4) recently described nine eIF2BΔ mutations that yielded a phenotype similar to that observed in cells lacking eIF2Bα, i.e. increased eIF2α phosphorylation was not accompanied by reduced cell growth rates and increased GCN4 translation. The results suggested that, like eIF2B lacking the α-subunit, eIF2B with any one of these point mutations in the δ-subunit should be resistant to inhibition by eIF2α(P). Four of the amino acids that were found to be mutated in yeast eIF2BΔ are conserved in the amino acid sequence of the rat protein (4). Of the conserved residues, substitutions of Glu177 and Leu81 with Lys and Gln, respectively, yielded a phenotype exhibiting the least apparent sensitivity to substrate phosphorylation. When a double mutation was made in yeast eIF2BΔ combining these two substitutions, the phenotype observed was identical to the phenotype of the L381Q single mutation. Therefore, in the present study, the same double mutation was made in rat eIF2BΔ. The activity of eIF2B was measured as the exchange of [3H]GDP bound to eIF2 for free nonradiolabeled GDP with time as described under "Materials and Methods." The results represent the mean ± S.E. of three experiments. Within each experiment, two independent assays were performed for each condition.

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eIF2Bδ and the mutant protein was coexpressed with the other four wild-type subunits. Recombinant eIF2B containing the mutant δ-subunit (referred to hereafter as eIF2B(δ*)) was purified from Sf9 cells as described above for wild-type eIF2B. Unexpectedly, over 50% of the mutant eIF2Bδ was degraded during the purification procedure, even in the presence of a mixture of eight different protease inhibitors. Because of the difficulty in obtaining purified eIF2B(δ*) with equimolar amounts of all five subunits, the exchange activity of eIF2B(δ*) was assessed in cell extracts rather than with the purified protein. As shown in the inset to Fig. 5, no degradation of eIF2Bδ occurred during preparation of extracts from cells expressing either the wild-type or mutant protein. It can also be seen that the amount of expressed protein was essentially the same for each of the five. As observed using purified eIF2B, incubation of extracts of Sf9 cells expressing all five wild-type eIF2B subunits with eIF2α(p) prior to assay (Fig. 5, open symbols) significantly reduced exchange activity compared with extracts incubated with unphosphorylated substrate (Fig. 5, closed symbols). In addition, preincubation of extracts of cells expressing eIF2B(δ*) with either eIF2α(p) or eIF2δ resulted in little difference in exchange activity. Similar to the results observed for eIF2B(δ*), the exchange activity of eIF2B(δ*) was only minimally inhibited by phosphorylation with eIF2α(p). The results show directly for the first time that both the α- and δ-subunits of eIF2B are important in mediating the inhibition of exchange activity by eIF2α(p).

Finally, the exchange activity of eIF2B(δ*) was examined using phosphorylated eIF2α as substrate. As seen in Fig. 6, both wild-type eIF2B and eIF2B(δ*) catalyzed GDP exchange using eIF2α(p) as substrate (open symbols), although the activity was substantially lower using phosphorylated compared with unphosphorylated eIF2α (closed symbols). However, unlike eIF2B lacking the α-subunit, the exchange activity using phosphorylated eIF2α as substrate was nearly the same for eIF2B(δ*) as for wild-type eIF2B. Thus, although eIF2B(δ*) was less sensitive to the inhibitory effect of eIF2α(p) on nucleotide exchange using unphosphorylated eIF2α as substrate (Fig. 5), it remained largely incapable of using phosphorylated eIF2α as substrate.

**DISCUSSION**

Regulation of translation initiation through phosphorylation of the α-subunit of eIF2α occurs in response to a variety of stimuli including deprivation of amino acids (10–12), glucose (12), purines (13), or serum (12). Phosphorylation of eIF2α does not directly inhibit formation of either the eIF2-GTP-Met-tRNAi ternary complex or the 43 S preinitiation complex (i.e. a 40 S ribosomal subunit associated with eIF-1A, eIF-3, and the eIF2 ternary complex), as these reactions proceed efficiently in vitro with the phosphorylated factor (14). Instead, phosphorylation of the α-subunit of eIF2 is thought to impede eIF2B activity by sequestering eIF2B into an inactive complex. Three lines of evidence support this assumption. (a) eIF2B reportedly does not catalyze GDP exchange on eIF2α(p) (15); (b) eIF2α(p) displaces unphosphorylated eIF2 bound to eIF2B but unphosphorylated eIF2 does not (16); and (c) eIF2α(p) inhibits the activity of eIF2B in the presence of low concentrations of substrate (i.e. <10-fold molar excess of eIF2-GDP to eIF2α(p))) but at higher substrate concentrations the inhibition caused by eIF2α(p) is negligible (15), suggesting that eIF2α(p) is acting as a competitive inhibitor of eIF2B.

Phosphorylation of eIF2α plays a critical role in the general control response in *S. cerevisiae*. Starvation of *S. cerevisiae* for any one of at least 10 amino acids leads to phosphorylation of eIF2α and increased translation of the mRNA coding for the transcription factor GCN4 (reviewed in Refs. 6 and 7). The latter effect is dependent upon the presence of eIF2B (reviewed in Refs. 7 and 17). In yeast, it has been suggested that three of the five subunits of eIF2B are involved in recognition of the phosphorylation status of eIF2α. In particular, deletion of...
eIF2Ba has no effect on cellular growth under nonstarvation conditions (18). However, eIF2Ba is required for induction of GCN4 translation under amino acid starvation conditions (19) and the induction of GCN4 is dependent upon phosphorylation of eIF2α (20). Moreover, deletion of eIF2Ba reduces the growth-inhibitory effect of high level eIF2 phosphorylation catalyzed by overexpression of the human double-stranded RNA-activated eIF2α protein kinase, PKR (21). A more recent study has identified point mutations in the α-subunit of eIF2B that are even more effective than deletion of the subunit in reversing the effects of eIF2 phosphorylation on translation and growth (4). These results suggest that the primary function of the α-subunit of eIF2B is to mediate the inhibitory effects of eIF2α phosphorylation on eIF2B catalyzing guanine nucleotide exchange on eIF2B results suggest that the phenotype observed in yeast lacking properties of yeast eIF2B are similar to the rat protein, the ing a mutant form of the expressing the five wild-type eIF2B subunits and cells express- guanine nucleotide exchange was similar in extracts of Sf9 cells activity of eIF2B in response to eIF2 inhibition of high level eIF2 phosphorylation catalyzed results suggest that the primary function of the α-subunit of eIF2B is to mediate the inhibitory effects of eIF2α phosphorylation on eIF2 as substrate. This result suggests that the affinity for eIF2α (P) is significantly less for the mutant forms of eIF2B than for the wild-type protein. In summary, the present study provides the first biochemical demonstration that both the α- and δ-subunits of eIF2B play important roles in regulating the guanine nucleotide exchange activity of the protein in response to phosphorylation of eIF2α. In particular, eIF2B lacking the α-subunit or containing a mutant form of the δ-subunit is completely resistant to inhibition by eIF2α (P). Finally, lack of the α-subunit allows for faster GDP exchange using eIF2α (P)[3H]GDP as substrate. Acknowledgment—We acknowledge the excellent technical assistance of Lynne Hugendubler.

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