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A Region Rich in Aspartic Acid, Arginine, Tyrosine, and Glycine (DRYG) Mediates Eukaryotic Initiation Factor 4B (eIF4B) Self-Association and Interaction with eIF3

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The binding of mRNA to the ribosome is mediated by eukaryotic initiation factors eukaryotic initiation factor 4F (eIF4F), eIF4B, eIF4A, and eIF3. eIF4F binds to the mRNA cap structure and, in combination with eIF4B, is believed to unwind the secondary structure in the 5′ untranslated region to facilitate ribosome binding. eIF3 associates with the 40S ribosomal subunit prior to mRNA binding. eIF4B copurifies with eIF3 and eIF4F through several purification steps, suggesting the involvement of a multisubunit complex during translation initiation. To understand the mechanism by which eIF4B promotes 40S ribosome binding to the mRNA, we studied its interactions with partner proteins by using a filter overlay (protein-protein [far Western]) assay and the two-hybrid system. In this report, we show that eIF4B self-associates and also interacts directly with the p170 subunit of eIF3. A region rich in aspartic acid, arginine, tyrosine, and glycine, termed the DRYG domain, is sufficient for self-association of eIF4B, both in vitro and in vivo, and for interaction with the p170 subunit of eIF3. These experiments suggest that eIF4B participates in mRNA-ribosome binding by acting as an intermediary between the mRNA and eIF3, via a direct interaction with the p170 subunit of eIF3.

Initiation of translation in eukaryotes is thought to occur by binding of the 40S ribosomal subunit at or near the cap structure of an mRNA, followed by ribosome scanning of the 5′ untranslated region until the initiator AUG is encountered (28). Initiation of translation requires the assembly of macromolecular complexes, both on the 40S ribosome and on the mRNA. Eukaryotic initiation factors 1A and 3 (eIF1A and eIF3) associate with the 40S ribosomal subunit prior to the mRNA binding step. eIF1A and eIF3 inhibit joining of the 40S and 60S ribosomal subunits in the absence of mRNA (7, 17, 32). The next event in the assembly of translation-competent 40S ribosomal subunits is the binding of a ternary complex composed of eIF2, GTP, and Met-tRNA$_i$, an association which is stabilized by eIF3 (8, 17, 36). This complex is referred to as the 43S preinitiation complex.

A prerequisite for binding of the mRNA to the 43S preinitiation complex is thought to be the interaction of several translation initiation factors with the mRNA. The first translation factor to make contact with the mRNA is eIF4F. This factor is a heterotrimer comprised of (i) eIF4E, a 24-kDa protein which binds the cap structure present at the 5′ end of all cellular (except organellar) mRNAs (41); (ii) eIF4A, a 50-kDa polypeptide which is a bidirectional RNA helicase (37, 39); and (iii) eIF4G (formerly p220), a 150-kDa protein to which both eIF4E and eIF4A bind directly (21, 23). eIF3 has also been shown to interact with the carboxy terminus of eIF4G and has thus been postulated to serve as a link between the mRNA and the 40S ribosome (21).

eIF4B facilitates the binding of the mRNA to the 43S preinitiation complex, as demonstrated by reconstitution studies of an in vitro translation system (43). eIF4B is an RNA-binding protein with a canonical RNA recognition motif (RRM) (for reviews, see references 10, 24, and 31) near its amino terminus and an arginine-rich RNA binding region in the carboxy terminus (27, 33). The arginine-rich region binds RNA nonspecifically, while the RRM binds specifically to 18S rRNA (26, 27). eIF4B can associate with a specific RNA target and a nonspecific RNA simultaneously, suggesting that it may serve to facilitate the binding of the 40S ribosomal subunit to the mRNA by acting as a bridge between the mRNA and the 18S rRNA (27). Besides its RNA binding activity, eIF4B stimulates the ATPase (1, 18) and RNA helicase (37, 39) activities of eIF4A. A functional interaction between eIF4A and eIF4B in vivo is also evident, since yeast eIF4B, expressed from a multicopy plasmid, is able to complement a temperature-sensitive mutant of eIF4A (12). More recently, mammalian and yeast eIF4Bs were shown to possess RNA annealing activity (3). It is thought that eIF4F, properly positioned on the mRNA near the cap structure via the eIF4E subunit, unwinds RNA secondary structure in the 5′ untranslated region of the mRNA, in conjunction with eIF4B, to create a site accessible for ribosome binding (40). This model is consistent with the observation that secondary structure in the 5′ untranslated region of an mRNA is inhibitory to translation (4, 35). Furthermore, an mRNA with extensive 5′ untranslated region secondary structure is better translated in cells that overexpress eIF4E (20), and it is poorly translated in a yeast strain that has been disrupted for the eIF4B gene, *TIF3* (2).

To gain further understanding into how eIF4B promotes binding of mRNA to the 40S ribosomal subunit, we examined protein-protein interactions mediated by eIF4B. We demonstrate that recombinant eIF4B self-associates in vitro and in vivo. We mapped the homotypic interaction site of eIF4B to a 99-amino-acid region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG-rich region [30] or DRYG domain). Filter overlay (protein-protein [far Western]) assays (9) of cell extracts reveal that eIF4B interacts with the p170 subunit of eIF3 and that this interaction is also mediated by the DRYG domain.

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MATERIALS AND METHODS

Vectors and proteins. The construction of pGEX4B, -N367, -N355, -N312, -N250, -N171, -NΔ253, and -Δ230-247 has been previously described (26). pGEXN213 was obtained by digesting pGEX4B with *Eco*NI and *Sma*I. The vector-containing fragment was blunt ended with the Klenow fragment of *Escherichia coli* DNA polymerase and religated. pGEXNΔ180 and -NΔ352 were generated by exonuclease III-mung bean nuclease digestion as described previously (26). pGEX 180-312 was created by digesting pGEXNΔ180 with *Sac*I and *Sma*I. The vector-containing fragment was blunt ended and religated. pGEX2THMK 213-312 was obtained by digesting pGEX4B with *Eco*NI and *Sac*I. The 300-bp fragment was blunt ended and ligated into pGEX2T[128/129] (9) that had been linearized with *Eco*RI and blunt ended. pGEX2THMK-4B was constructed by excising the *Bam*HI fragment from pGEX4B, blunt ending it, and ligating it into pGEX2T[128/129] that had been linearized with *Eco*RI and blunt ended. Because of the leakiness of the *tac* promoter and the extensive degradation of the resulting glutathione *S*-transferase (GST)–eIF4B fusion protein, the GST-FLAG-heart muscle kinase (HMK)-eIF4B fusion was put under the control of the T7 promoter. An *Nde*I site was created at the initiator ATG codon of the GST cDNA by PCR, using the primers AACAGTACATATGTCCCCTATAC TAG and CTGTGCCAAGTGGTCG and using pGEX-4B as the template. The PCR product was digested with *Nde*I and *Bst*XI and ligated to the *Bst*XI-*Pst*I fragment excised from pGEX4B and to pET3b (42) that had been cut with *Nde*I and *Pst*I. The resulting vector, pET2THMK-4B, generates a GST-FLAG-HMK fusion protein with eIF4B which is under the control of the T7 promoter. The GST-HMK-eIF4B fusion protein was overexpressed in *E. coli* K38 cells, and induction was performed by heat shock at 42°C for 30 min. All GST-HMK-eIF4B fragments were purified on glutathione-Sepharose (Pharmacia). GST-HMK-eIF4B wild type (wt) was further purified on a heparin EconoPak column (Bio-Rad) as described previously for GST-eIF4B (26). For the yeast two-hybrid system expression vectors, we used pGBT9 and pGADGH, which carry the GAL4 DNA binding domain (GAL4-DB) and the GAL4 transactivator domain (GAL4-TA), respectively (gifts from P. Bartel, State University of New York, Stony Brook, and G. Hannon, Cold Spring Harbor Laboratory). pGBT9-4B was constructed by cutting pGEX-4B with *Bam*HI. The excised eIF4B DNA was ligated in pGBT9 linearized with *Bam*HI. For pGADGH-4B, the *Bam*HI fragment from pGEX-4B was blunt ended with the Klenow fragment of *E. coli* DNA polymerase and ligated into pGADGH digested with *Sma*I. To generate N312 GAL4-DB fusions, pGBT9-4B was digested with *Sac*I or *Eco*NI and with *Pst*I. Similarly, N312 GAL4-TA fusions were generated by cutting pGADGH-4B with *Sac*I or *Eco*NI and with *Sal*I. Following blunt ending with T4 DNA polymerase, the vector-containing fragments were religated. Recombinant eIF4B was purified as described previously (34).

Far Western analysis. Purified GST-HMK-eIF4B fusions (1 to 3 μg) were ^{32}P-labeled by using HMK (Sigma) as described previously (9). *E. coli* extracts expressing various fragments of eIF4B were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Millipore). Immobilized proteins were denatured by incubating the membranes with 6 M urea in HBB buffer (25 mM *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 25 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol). The proteins were renatured in situ by a progressive dilution of urea in HBB buffer. Although not necessary, we found that these denaturation-renaturation steps improved the sharpness of the signal. The membranes were blocked with 5% milk in HBB and incubated overnight in hybridization buffer (20 mM HEPES-KOH [pH 7.5], 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1% milk) containing the ^{32}P-labeled probe at 250,000 cpm/ml and unlabeled purified GST at 1 μg/ml. The membranes were washed three times with hybridization buffer and processed for autoradiography.

Western blotting (immunoblotting). Proteins were resolved on SDS-polyacrylamide gels and blotted onto nitrocellulose or PVDF membranes. Membranes were blocked for 1 h at 25°C in TBST (Tris-buffered saline, 0.1% Tween 20) containing 5% powdered milk. Antibodies were diluted in TBST with either 1% bovine serum albumin (BSA) or 1% powdered milk and incubated with the membrane for 1 h at 25°C. The following dilutions of antibodies were used: monoclonal anti-eIF4B antibodies 9E11 and 12B1, 1:100 and 1:500, respectively; monoclonal anti-eIF3 p170 antibody (25), 1:10; rabbit polyclonal anti-eIF4B antibody, 1:1,000; and goat polyclonal anti-eIF3 antibody (gift from J. Hershey), 1:3,000. Membranes were washed in TBST and incubated 30 min with horseradish peroxidase-conjugated secondary antibodies as follows: for monoclonal anti-eIF4B, anti-mouse immunoglobulin G (IgG); for polyclonal anti-eIF4B, anti-rabbit IgG; for anti-p170, anti-mouse IgM; and for anti-eIF3; anti-goat IgG. Immunoreactive species were visualized by chemiluminescence (ECL system; Amersham).

Two-hybrid system analysis. *Saccharomyces cerevisiae* Y526 (5) was transformed with various combinations of the yeast expression constructs by the lithium acetate method (16). Identification of colonies with a reconstituted GAL4 activity was performed by a 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal) colony filter assay. Colonies were replica plated onto a nitrocellulose filter (Hybond-N; Amersham) and permeabilized by immersion in liquid nitrogen. The filters were placed on Whatman 3MM paper which had been soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) containing 1 mM X-Gal and incubated overnight at 37°C to allow for color development. For quantitative β-galactosidase assays, randomly picked colonies were grown in liquid selection medium before the cells were harvested and lysed with glass beads. β-Galactosidase was quantitated as described previously (38). Background β-galactosidase activity was 0.63 nmol/min/mg. Expression of GAL4-DB and GAL4-TA fusion proteins was monitored by Western blotting. Cells (3×10^7) were lysed with glass beads in Laemmli buffer, loaded on an SDS–12% polyacrylamide gel, and transferred to a PVDF membrane. Fusion proteins were detected by using an antibody raised against GAL4 which detects both GAL4-BD and GAL4-TA (a generous gift from James Hopper, Pennsylvania State University).

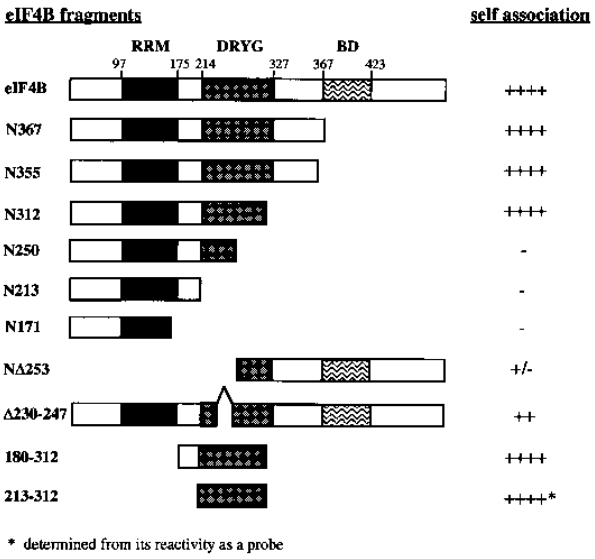

FIG. 1. Schematic representation of eIF4B fragments. The RRM, the DRYG domain, and the carboxy-terminal RNA binding site, or basic domain (BD), are indicated. Also shown is the self-association activity of each of these fragments, as determined by far Western analysis. ++++, 100% activity; ++, 25 to 50% activity; +/−, 5% activity; −, no detectable activity.

Immunoprecipitations. HeLa R19 cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 75 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100). Nuclei and cellular debris were removed by centrifugation. Antibodies (2 μl of anti-eIF4B polyclonal antibody 706, 2 μl of preimmune 706 serum, and 5 μl of anti-eIF4B monoclonal antibody 9E11) were added to 200 μg of cell extract and incubated on ice for 30 min. A 50% suspension (50 μl) of protein A-Sepharose (Repligen) that had been washed in lysis buffer and preincubated with BSA was added to the mixture and incubated end over end at 4°C for 90 min. The beads were washed six times with 1 ml of lysis buffer and boiled in Laemmli buffer to which 100 mM of β-mercaptoethanol had been freshly added. Proteins were resolved on SDS–8 or 11% polyacrylamide gels and transferred onto PVDF membranes. Immunoblotting was performed as described above.

RESULTS

The self-association site of eIF4B maps to the DRYG domain. Human eIF4B is a 69-kDa protein that migrates aberrantly on SDS-polyacrylamide gels at 80 kDa (30). The molecular mass of rabbit reticulocyte eIF4B, as determined by gel filtration chromatography in the presence of 0.5 M KCl, is 220 kDa, which led to the suggestion that eIF4B is an asymmetric dimer (18). Previous structure-function analyses, using a series of truncated eIF4B proteins, identified regions responsible for sequence-nonspecific and -specific RNA binding and eIF4A helicase stimulation (26, 27, 33). eIF4B fragments were used in this study to map the self-association region of eIF4B by a filter overlay assay (9). Figure 1 illustrates the various fragments which were used and summarizes the results. *E. coli* crude extracts expressing different C-terminal truncations of eIF4B were resolved on SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with a ^{32}P-labeled GST-eIF4B

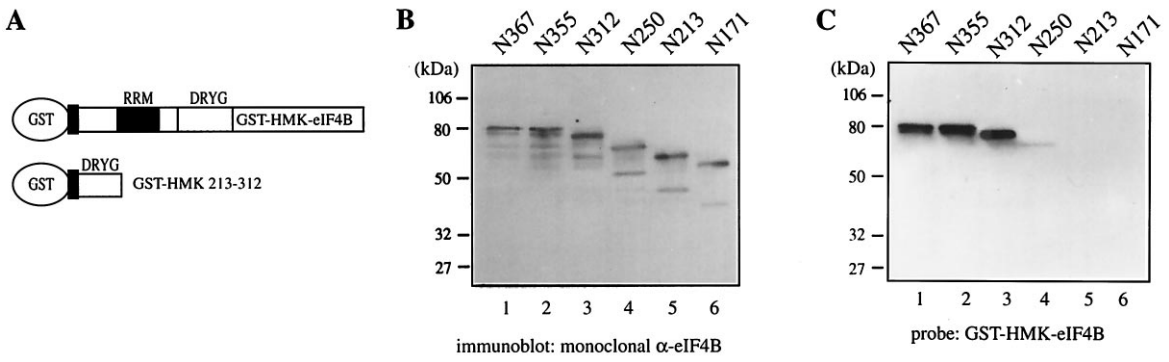

FIG. 2. Mapping of the eIF4B self-association site. (A) GST-HMK probes used in this study. The HMK phosphorylation site is represented by a grey rectangle. Also shown are the RRM and the DRYG domain. (B) Immunoblot analysis using anti-eIF4B monoclonal antibody 12B1 (α-eIF4B) on *E. coli* extracts expressing various fragments of eIF4B. (C) Far Western analysis on a membrane similar to that shown in panel B, with ^{32}P-labeled GST-HMK-eIF4B as a probe.

fusion protein (eIF4B probe) tagged with the HMK phosphorylation site (Fig. 2A, upper construct). To monitor background dimerization due to potential GST-GST interactions (44), duplicate filters were probed with HMK-tagged GST. Background was minimized by the addition of a large excess of unlabeled GST protein in the hybridization buffer. The different GST-eIF4B proteins were expressed to similar levels, as determined by Western blotting using a monoclonal antibody directed against the amino terminus of eIF4B (Fig. 2B). All fragments of eIF4B retaining the amino-terminal 312 amino acids of the protein were capable of interacting with the eIF4B probe (Fig. 2C, lanes 1 to 3; data for eIF4B wt are shown in Fig. 3B). Further deletions generated eIF4B fragments that were unable to associate with the eIF4B probe (fragments N250, N213, and N171 [lanes 4 to 6]). (The faint band seen for N250 is artifactual since it does not migrate exactly with the N250 protein [compare lanes 4 in Fig. 2B and C] and is not reproducible.) ^{32}P-labeled GST-HMK failed to interact with any of the fragments of eIF4B (data not shown), excluding the possibility that the signal arose from GST-GST interaction. The results suggest that the self-association domain of eIF4B lies between amino acids 213 and 312. Amino acids 213 to 312 encompass the DRYG domain of eIF4B. To determine whether this region can mediate homotypic interactions independently from other regions of eIF4B, four kinds of experiments were performed: (i) a fragment encompassing amino acids 180 to 312 was tested for its ability to interact with eIF4B, (ii) an amino-terminal deletion fragment (NΔ253) that contains the carboxy-terminal half of the DRYG domain was tested for interaction with GST-HMK-eIF4B to determine whether amino acids 254 to 312 of the DRYG domain are capable of supporting eIF4B self-association, (iii) a GST-HMK fusion encompassing amino acids 213 to 312 of eIF4B was used as a probe in the filter overlay assay, and (iv) amino acids 230 to 247 were removed from the DRYG domain to create the Δ230-247 mutant. The relative amounts of GST-eIF4B wt, Δ230-247, 180-312, and NΔ253 were estimated by Western blotting with a polyclonal antibody against eIF4B (Fig. 3A; note that the level of fragment 180-312 may be underestimated because of the potential loss of antigenic epitopes). When an identical blot was probed with ^{32}P-labeled GST-HMK-eIF4B, a threefold decrease in the ability of Δ230-247 to interact with the probe, relative to eIF4B wt, was observed (Fig. 3B; compare lanes 1 and 2). Fragment 180-312 reacted with the probe as well as eIF4B wt (lane 3), while an interaction with NΔ253 was not detectable (lane 4). Upon a longer exposure, however, a weak association between NΔ253 and the GST-HMK-eIF4B probe was detected (data not shown). Another fragment (NΔ352) was unable to interact with eIF4B (data not shown). The GST–HMK–213-312 probe (Fig. 2A, lower construct) interacted with various eIF4B truncations similarly to full-length eIF4B (Fig. 3C). Taken together with the previous results, these experiments clearly demonstrate that the region between amino acids 213 and 312 is necessary and sufficient to mediate eIF4B homotypic interactions. The inability of NΔ253 to react with the probe at levels comparable to wt levels suggests that

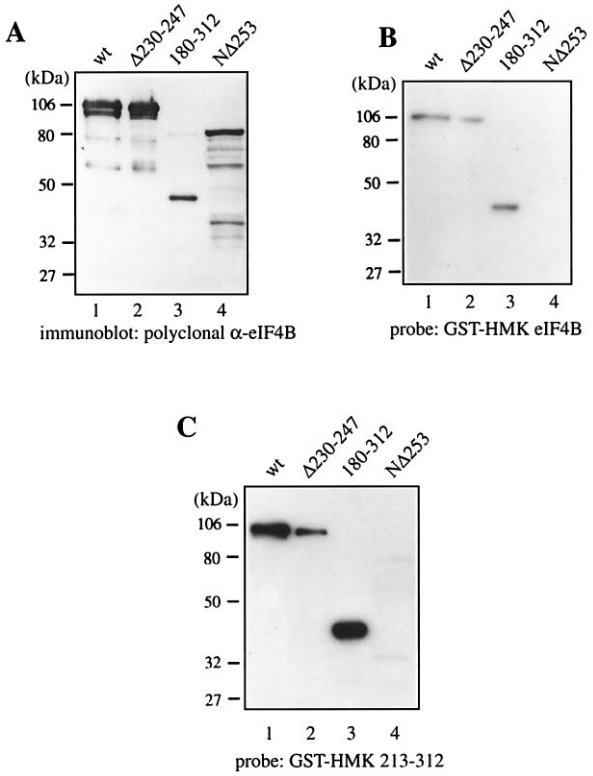

FIG. 3. The DRYG domain is sufficient to mediate self-association. (A) Immunoblot analysis using anti-eIF4B polyclonal antibody 706 (polyclonal α-eIF4B) on *E. coli* extracts expressing the following GST-eIF4B fusions: wt, Δ230-247, 180-312, and NΔ253. (B) Far Western analysis on a membrane similar to that shown in panel A, with ^{32}P-labeled GST-HMK-eIF4B as a probe. (C) Same as panel B except that ^{32}P-labeled GST–HMK–213-312 was used as a probe.

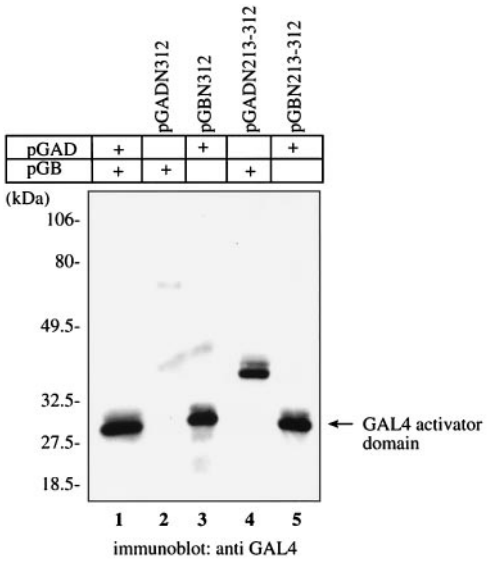

FIG. 4. Expression of GAL4-eIF4B fusion proteins used in the two-hybrid system analysis. Yeast cells (3×10^7) transformed with pGAD-eIF4B and pGBT9 or with pGB-eIF4B and pGADGH were lysed with glass beads in Laemmli buffer and blotted onto a nitrocellulose membrane. GAL4 fusion protein levels was determined by using an antibody directed against GAL4, which can recognize both GAL4-TA and GAL4-DB.

amino acids 254 to 312 alone are not sufficient to mediate an interaction. Also, amino acids 230 to 247 contribute to the self-association function, either by allowing proper folding of the DRYG domain or by self-associating directly.

eIF4B self-associates in vivo. The results obtained by far Western analysis were further supported by the yeast two-hybrid assay (15). Fragments of eIF4B (N312 and 213-312) were expressed as both GAL4-TA and GAL4-DB fusion proteins, and a series of combinatorial pairs was tested for the ability to reconstitute GAL4 transactivation activity. The level of expression in yeast cells was determined by Western blotting using a polyclonal antibody directed against GAL4 (Fig. 4). Both GAL4-TA fusions were detected (lanes 2 and 4). On the other hand, neither the GAL4-DB fusion proteins nor the GAL4-DB fusion proteins generated by the pGBT9 vector were detected. After a very long exposure, however, very small amounts of GAL4-DB fusion proteins could be seen (data not shown). The reasons for the low expression levels of GAL4-DB fusion proteins are unknown, but they could be due to the instability of the proteins. Table 1 summarizes the levels of β-galactosidase activity obtained from several experiments. N312 homodimerized and dimerized with 213-312, yielding β-galactosidase activities 301- and 10.3-fold, respectively, over the background level. These data are asymmetric, as N312, but not the 213-312 fragment, expressed as a GAL4-TA fusion could dimerize with 213-312. A directionality with results of protein-protein interaction assays has often been observed with the two-hybrid system (e.g., reference 14). This experiment shows that the DRYG-mediated protein-protein interaction occurs in vivo and confirms the importance of the DRYG domain for self-association.

eIF4B interacts directly with eIF3. eIF4B copurifies through many purification steps with eIF3 and eIF4F and is separated from these factors by high salt (0.5 M KCl). This interaction presumably has biological relevance. To determine if eIF4B associates directly with eIF3 and eIF4F and to further study these interactions, a filter overlay assay was performed with

TABLE 1. Two-hybrid system

DNA binding (pGADGH)	Transactivation (pGBT9)	Colony color	β-Galactosidase activity[a]	
			nmol/min/mg	Fold over background
N312	Empty	White	0.60	1.1
Empty	N312	White	0.92	1.5
213-312	Empty	White	0.53	0.8
Empty	213-312	White	0.39	0.6
N312	N312	Blue	190.00	301.0
N312	213-312	White	0.55	0.9
213-312	N312	Light blue	6.51	10.3
213-312	213-312	White	0.72	1.1

[a] Values for β-galactosidase activity were derived from at least three independent assays. The background level used for calculations was derived from the average β-galactosidase activity obtained with N312 and 213-312 fusions tested against the corresponding empty vector and was 0.63 nmol/min/mg.

various cell extracts, using different eIF4B fragments as probes. GST-HMK-eIF4B bound to two major proteins of approximately 170 and 100 kDa and also associated weakly with a protein of 80 kDa in a HeLa extract and reticulocyte lysate (Fig. 5A, lanes 1 and 2). The 100-kDa protein was absent from rabbit reticulocyte lysates (lane 2). In Krebs extracts, an additional protein of 110 kDa was present (lane 3). The 80-kDa polypeptide comigrated with recombinant eIF4B (lane 4) and thus is probably eIF4B. When an identical blot was probed with GST–HMK–213-312, only the 170- and 80-kDa proteins reacted (Fig. 5B). Thus, the DRYG domain does not interact with the 100- and 110-kDa proteins. A control blot probed with GST-HMK did not yield any signal (data not shown).

We sought to determine the identity of the 170-kDa protein. eIF3 is a multisubunit translation initiation factor which is composed of 10 polypeptides, including a 170-kDa polypeptide, p170 (6, 19a, 29). Because eIF4B was reported to associate with eIF3 in cell extracts, we examined the possibility that the 170-kDa polypeptide is the high-molecular-weight subunit of eIF3. To this end, a preparation of eIF3 (generous gift from H. Trachsel) was resolved on an SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Immunoblot analysis using a monoclonal antibody directed against the p170 subunit of eIF3 (25) shows the presence of the intact p170, as well as some degradation products (Fig. 5C). Probing a duplicate membrane with ^{32}P-labeled GST–HMK–213-312 revealed proteins of 170 and 80 kDa in HeLa cell and rabbit reticulocyte extracts (Fig. 5D, lanes 1 and 2). Purified eIF3 contained a strongly reactive polypeptide migrating at 170 kDa (lane 3). Taken together, these results demonstrate that eIF4B associates directly with the p170 subunit of eIF3 and that this interaction is mediated by the DRYG domain.

To further substantiate the conclusion that eIF4B interacts with eIF3, coimmunoprecipitation experiments were performed. eIF4B was immunoprecipitated from HeLa cell extracts by using either anti-eIF4B polyclonal antibody 706, preimmune 706 serum, or a monoclonal antibody that recognizes the carboxy-terminal end (9E11). The immunoprecipitates were blotted onto nitrocellulose and probed with antibodies directed against various proteins. eIF4B was precipitated with both anti-eIF4B antibodies (Fig. 6A, lanes 2 and 4), whereas no protein was precipitated with the preimmune serum (lane 3). Approximately 30% of the total eIF4B was recovered in the immunoprecipitate. eIF3 coimmunoprecipitated efficiently with eIF4B, as judged from the large amount present in the immune complex relative to the total extract (10% of eIF3 input was recovered [Fig. 6B; compare lanes 1, 2, and 4]). This

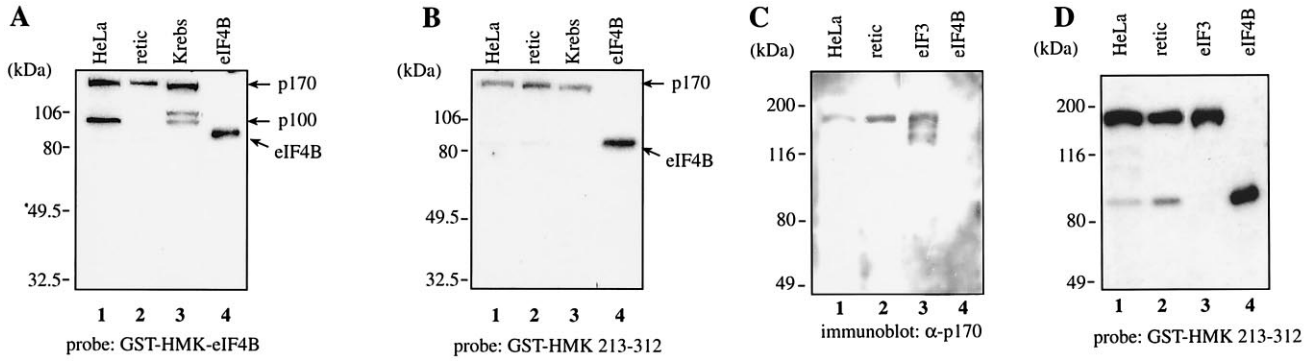

FIG. 5. The DRYG domain of eIF4B interacts with the p170 subunit of eIF3. (A) Far Western analysis on cytoplasmic extracts (75 μg) using ^{32}P-labeled GST-HMK-eIF4B as a probe. Recombinant eIF4B (0.5 μg) was used as a positive control. The positions and molecular masses of interacting proteins are indicated by arrows. (B) Same as panel A except that ^{32}P-labeled GST–HMK–213-312 was used as a probe. (C) Western blot analysis using a monoclonal antibody directed against the p170 subunit of eIF3 (α-p170). (25). (D) Far Western analysis on a membrane similar to that shown in panel C. ^{32}P-labeled GST–HMK–213-312 was used as a probe. retic, reticulocyte.

result is consistent with a direct interaction between eIF4B and the p170 subunit of eIF3. As a negative control, the presence in the immune complexes of proteins not known to be related to eIF4B function was examined. Actin (Fig. 6C) and the La autoantigen (data not shown) were not found in the eIF4B immunoprecipitates.

DISCUSSION

The DRYG domain. In this study, we have mapped a self-association domain in eIF4B to the DRYG domain, located between amino acids 214 and 327. Residues 213 to 312 are sufficient for the interaction. Deletion of the carboxy-terminal-most part of the DRYG domain (mutants N250, N213, and N171) resulted in a complete loss of self association. Similarly, NΔ253, in which the carboxy-terminal-most part of the DRYG domain is intact, interacted with eIF4B very weakly. Removal of 18 amino acids in the DRYG domain (Δ230-247) led to a threefold decrease in self-association. These data suggest that amino acids between positions 250 and 312 are crucial for the interaction but not sufficient, since NΔ253, which still contains amino acids 254 to 312, showed virtually no activity, and the Δ230-247 deletion, which is outside amino acids 250 to 312, exhibited a decrease in self-association activity. It is possible that both of these deletions induce a conformational change which prohibits dimerization. Alternatively, a minimal amount of residues outside amino acids 250 to 312, or amino acids 250 to 254, is necessary for dimerization. There is also the possibility that two segments within the DRYG domain mediate self-association. The data, however, do not permit us to conclude whether the predominant species of eIF4B is a dimer, trimer, or tetramer.

How is self-association promoted? Programs such as that of Chou and Fasman (11) predict a very high content of random coil structure within the DRYG domain, probably as a result of the abundance of glycine residues. The binding energy may be provided by electrostatic interactions between the alternating positive and negative charges. However, glutaraldehyde cross-linking and gel filtration studies showed that eIF4B self-association is resistant to KCl concentrations of at least 500 mM (18, 25a). Homodimerization could be the result of hydrophobic interactions between the tyrosine residues, where phenyl rings may stack. Alternatively, self-association may be mediated by the formation of β-sheets between two eIF4B monomers. The DRYG domain of eIF4B does not show homology to any known proteins except for the SRYG repeats of the azoospermia factor AZF (22). This homology may be coincident rather than an indication of conservation. However, it may also represent a novel type of protein-protein interaction domain in which tyrosine residues play an important role.

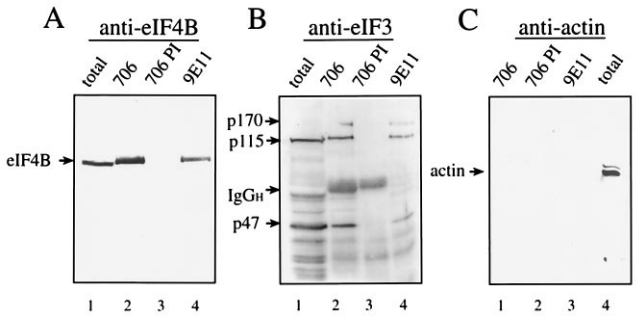

FIG. 6. eIF3 coimmunoprecipitates with eIF4B. Western blots of immunoprecipitates are shown. eIF4B from HeLa cell extracts (200 μg) was immunoprecipitated with polyclonal antibody 706 or monoclonal antibody 9E11. Preimmune 706 serum (706 PI) was used as a negative control. In panels A and B, the positions of three of the eIF3 subunits are indicated by arrows. For the lanes labeled "total," 30 μg of HeLa extract was loaded directly on the gel.

Possible functions of self-association. The role of self-association for eIF4B function is not immediately clear. Previous studies on eIF4B have shown that the carboxy-terminal NΔ253 fragment exhibits sequence-nonspecific RNA binding (26). Furthermore, the amino-terminal N250 fragment binds to a specific RNA substrate with an affinity comparable to that of eIF4B wt (27). Both of these fragments are severely deficient for self-association. The NΔ253 fragment of eIF4B is also fully active in cooperating with eIF4A for RNA binding and retains considerable eIF4A helicase-stimulatory activity (26). The partial loss of helicase-stimulatory activity associated with NΔ253 may be due to deletion of the RRM, as we have previously shown that point mutations in ribonucleoproteins 1 and 2 reduce the helicase-stimulatory activity (26). The eIF4B fragment N312 (dimerization positive, helicase stimulation negative) does not exhibit any *trans*-dominant negative activity in the RNA helicase assay (25a). These results suggest that eIF4B self-interaction is not required for RNA binding and is not absolutely necessary for helicase-stimulatory activity.

We used several deletions in the DRYG domain to determine whether the self-association region of eIF4B and the

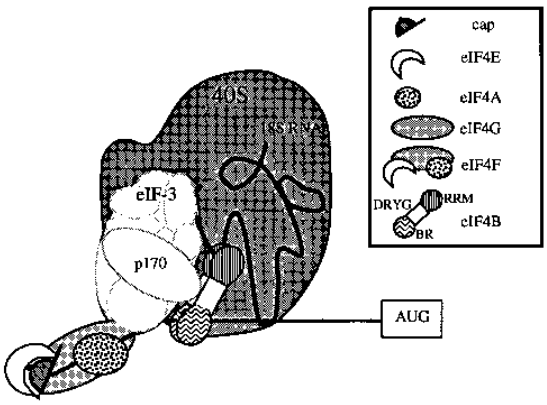

FIG. 7. Model for binding of the 40S ribosomal subunit to an mRNA. Four types of interactions may stabilize the mRNA-ribosome complex: (i) eIF3-eIF4G, (ii) eIF4B-eIF3, (iii) eIF4B-18S rRNA, and (iv) mRNA-rRNA. BR, basic region.

p170 interaction domain overlap. All of the truncations that abolished eIF4B self-interaction also abolished eIF3 binding (25a). Two possibilities may explain these results: (i) the self-association and p170 interaction sites overlap, in which case eIF4B homodimerization would prevent p170 interaction; and (ii) eIF4B must dimerize in order to generate a site for p170 binding. We cannot at present distinguish between these two possibilities because expression systems for intact p170 are not available. However, it is possible that a large proportion of the GST–HMK–213-312 probe already exists as a dimer (or multimer). This might explain why the eIF4B self-association signal in a HeLa extract is weaker than the p170 interaction signal (eIF4B and eIF3 are present at approximately 0.5 and 1 molecule, respectively, per ribosome in HeLa cells [13, 25]). Furthermore, the p170 protein does not contain a region homologous to the DRYG domain (25a), which is not consistent with a shared interaction domain between eIF4B and p170. On the other hand, overexpression of eIF4B in COS cells results in a general reduction of translation, a phenomenon that remains unexplained (30, 33). Removal of the DRYG domain abolishes the ability of eIF4B to inhibit translation (33). This could be consistent with the notion that eIF4B self-association prevents interaction with eIF3. However, some deletion mutants that retained the DRYG domain did not inhibit translation when overexpressed, while others that lacked the DRYG domain were strong translational inhibitors (33). Thus, the translational block induced by overexpression of eIF4B cannot be attributed solely to its ability to self-associate or interact with eIF3.

Our findings have implications for the mechanism of ribosome binding to mRNA. It was previously postulated that eIF4B serves as a bridge between the mRNA and the 40S ribosomal subunit by virtue of its ability to bind both mRNA and 18S rRNA (3, 27). The direct interaction between eIF3, which is present on the 43S preinitiation complex prior to mRNA binding, and eIF4B provides an additional link between the mRNA and the ribosome (Fig. 7). The abundance of protein-protein and protein-RNA interactions during mRNA-ribosome binding ensures that the mRNA is recognized by ribosomes with high fidelity.

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