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The j-Subunit of Human Translation Initiation Factor eIF3 Is Required for the Stable Binding of eIF3 and Its Subcomplexes to 40 S Ribosomal Subunits *in Vitro**

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Christopher S. Fraser‡, Jennifer Y. Lee, Greg L. Mayeur, Martin Bushell§, Jennifer A. Doudna¶, and John W. B. Hershey‖

From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

Eukaryotic initiation factor 3 (eIF3) is a 12-subunit protein complex that plays a central role in binding of initiator methionyl-tRNA and mRNA to the 40 S ribosomal subunit to form the 40 S initiation complex. The molecular mechanisms by which eIF3 exerts these functions are poorly understood. To learn more about the structure and function of eIF3 we have expressed and purified individual human eIF3 subunits or complexes of eIF3 subunits using baculovirus-infected Sf9 cells. The results indicate that the subunits of human eIF3 that have homologs in *Saccharomyces cerevisiae* form subcomplexes that reflect the subunit interactions seen in the yeast eIF3 core complex. In addition, we have used an *in vitro* 40 S ribosomal subunit binding assay to investigate subunit requirements for efficient association of the eIF3 subcomplexes to the 40 S ribosomal subunit. eIF3j alone binds to the 40 S ribosomal subunit, and its presence is required for stable 40 S binding of an eIF3bgi subcomplex. Furthermore, purified eIF3 lacking eIF3j binds 40 S ribosomal subunits weakly, but binds tightly when eIF3j is added. Cleavage of a 16-residue C-terminal peptide from eIF3j by caspase-3 significantly reduces the affinity of eIF3j for the 40 S ribosomal subunit, and the cleaved form provides substantially less stabilization of purified eIF3–40S complexes. These results indicate that eIF3j, and especially its C terminus, play an important role in the recruitment of eIF3 to the 40 S ribosomal subunit.

Eukaryotic initiation factor 3 (eIF3)[1] was first isolated and purified as a high molecular weight complex from rabbit reticulocytes (1–3). The mammalian factor possesses a molecular mass of about 600 kDa and contains at least 12 nonidentical protein subunits, named in order of decreasing molecular weight as recommended (4): eIF3a, eIF3b, eIF3c, eIF3d, eIF3l, eIF3e, eIF3f, eIF3g, eIF3h, eIF3i, eIF3j, and eIF3k (5, 6). Specific functions for mammalian eIF3 have been identified by a variety of *in vitro* experiments. It binds directly to 40 S ribosomal subunits in the absence of other initiation components (1), and affects the association/dissociation of ribosomes (7–10). It promotes the binding of Met-tRNA$_i$ and mRNA to the 40 S ribosomal subunit (5), and binds directly to eIF1 (11), eIF4B (12), eIF4G (13, 14), and eIF5 (15). Clearly, eIF3 plays a central role in the initiation pathway, perhaps structurally organizing other translational components on the surface of the 40 S ribosomal subunit.

An eIF3 complex was first identified and isolated from *Saccharomyces cerevisiae* by employing either of two assay systems: stimulation of methionyl-puromycin synthesis based on mammalian assay components (16) and stimulation of protein synthesis in a heat-inactivated yeast lysate derived from a conditional mutant of eIF3b (17). Purification of eIF3 using an oligohistidine-tagged eIF3b identified a core of five subunits associated with eIF5 (18). The five core subunits, eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i, are all essential for yeast growth and are conserved in mammalian eIF3. Recently, eIF3j has been shown to associate loosely with the core eIF3 complex in yeast (19) and has been suggested to augment the stability of eIF3 and possibly play a role in the 40 S ribosomal subunit assembly pathway (20).

Given the similarities between other yeast and mammalian initiation factors, the structural differences observed with eIF3 are somewhat surprising. These discrepancies may be due in part to subtle differences in the strengths of various protein-protein interactions. It is likely that the true subunit composition of eIF3 will not be resolved until a functional protein complex is reconstituted from separated subunits. To further understand the structure and function of mammalian eIF3, we have utilized the baculovirus expression system to prepare human eIF3 subunits that have orthologs in *S. cerevisiae*. In this study, we overexpressed different combinations of eIF3 subunits, including FLAG-tagged subunits, and affinity-purified the subcomplexes by anti-FLAG affinity beads. This approach allows assembly of human eIF3 components *in vivo*, recovery of stable subcomplexes and determination of their functions in *in vitro* assays for initiation. It also has the potential to generate structural information about subunit-subunit interactions and to identify specific functions of individual subunits. We focus here on the function of eIF3j in promoting the binding of core subcomplexes and purified eIF3 to 40 S ribosomal subunits and its reduced activity following cleavage by caspase-3.

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‡ Present address: Dept. of Molecular and Cell Biology, and Howard Hughes Medical Institute, University of California, Berkeley, CA 94720.

§ Supported by Grant 063233/B/00/Z from the Wellcome Trust while in the laboratory of Dr. P. Sarnow. Present address: Dept. of Microbiology and Immunology, Stanford University School of Medicine, 299 Campus Dr., Stanford, CA 94305.

¶ Present address: Dept. of Molecular and Cell Biology, and Howard Hughes Medical Institute, University of California, Berkeley, CA 94720.

‖ To whom correspondence should be addressed: Dept. of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616. Tel.: 530-752-3235; Fax: 530-752-3516; E-mail: jwhershey@ucdavis.edu.

[1] The abbreviation used is: eIF3, eukaryotic initiation factor 3.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—Materials for tissue culture and DNA oligonucleotides are from Invitrogen; [^{35}S]methionine is from ICN; DNA-modifying enzymes are from New England BioLabs; anti-FLAG affinity beads are from Sigma. Unless otherwise stated, all other chemicals are from Sigma.

Cell Culture—Spodoptera frugiperda 9 (Sf9) cells were grown in 100-ml spinner flasks at 27 °C in Sf-900 II serum-free medium (Invitrogen). For experiments, cells were seeded at 1×10^7 cells in 100-mm dishes prior to infection with the recombinant viruses described in individual figure legends.

Construction of Recombinant Baculoviruses—Baculoviruses allowing the expression of single subunits of human eIF3 were constructed in derivatives of the pFASTBAC1 vector (Invitrogen). DNA oligonucleotides were designed, annealed, and ligated into pFASTBAC1 to produce FLAG-FASTBAC1. This vector includes an NcoI site containing an AUG codon upstream of the FLAG-tag sequence followed by an in-frame AUG within an NdeI site. In addition, pET-28c (Novagen) was digested with BglII and XhoI and ligated into pFASTBAC1 digested with BamHI and XhoI to produce pFASTpET.

Untagged eIF3 subunit expression constructs were created as follows. The cDNA for full-length eIF3i (GenBank™ nucleotide accession number U39067) (21) was excised from an untagged construct in pET-28c by digesting with XbaI and XhoI and ligating into the equivalent sites of pFASTpET. Similarly, the eIF3g sequence (accession no. U96074) was released from pET-T7p44 (22) by digesting with XbaI and XhoI and inserted into pFASTpET. The full-length cDNA of eIF3b (accession no. U62583), a kind gift from Nahum Sonenberg (McGill University), was modified by PCR to include NcoI and NdeI sites at the 5′-end of the construct by using the primer 5′-CCATGGGGCATATG-CAGGACGCGGAGAACGT-3′. This allowed the creation of an untagged eIF3b coding sequence when ligated between NcoI and XhoI in FLAG-FASTBAC1. Full-length eIF3a (accession no. D50929) was modified by PCR so that the full-length coding sequence could be released by digestion with NdeI and SalI. The digestion of FLAG-FASTBAC1 containing untagged eIF3b with NdeI and XhoI allowed the ligation of eIF3a that had been digested with NdeI and SalI to result in untagged eIF3a.

To create FLAG fusion proteins, constructs were generated as follows. eIF3g and eIF3j were excised from pET-NHp44 (22) and pET-NHp35 (22) respectively, by digestion with NdeI and XhoI and ligated into FLAG-FASTBAC1 at the equivalent sites. eIF3i was subcloned from pGEXp36 (21) into pET-28c by digesting with NdeI and EcoRI and the resulting construct digested with NdeI and XhoI and ligated into FLAG-FASTBAC1 at the equivalent sites. The PCR-modified eIF3b sequence was digested with NdeI and XhoI and inserted into the same restriction sites of FLAG-FASTBAC1, while the PCR-modified eIF3a was digested with NdeI and SalI and inserted into the NdeI and XhoI sites of FLAG-FASTBAC1.

The recombinant FASTBAC vectors above were recombined with baculovirus DNA using DH10BAC *E. coli* (Invitrogen) and the high molecular weight DNA ("bacmid") purified according to the manufacturer's guidelines. Sf9 cells were transfected with bacmid DNA by using the calcium phosphate method (Promega) and viral stocks were prepared by three-step growth amplification according to the manufacturer's guidelines. eIF3b-HMK-FLAG, eIF3j-HMK-FLAG, and His_6-eIF3c-Myc viruses were kind gifts from Hiroaki Imataka, Shigenobu Morino, and Nahum Sonenberg (McGill University).

Expression of eIF3 Subunits and Preparation of Cell Extracts—Sf9 cells (1×10^7) were infected with baculoviruses expressing a single FLAG-tagged subunit of eIF3 and/or untagged subunits of eIF3, as indicated in each experiment. The cells were grown for 24 h and then supplemented with 0.5 mCi of [^{35}S]methionine for an additional 36 h. Cells were harvested after placing on ice, by washing once with phosphate-buffered saline (50 mM sodium phosphate, pH 7.0, 150 mM NaCl) and scraping in 1 ml of Buffer A (20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, 10% glycerol). Following a 5-min incubation on ice with occasional vortexing, extracts were centrifuged for 10 min at 12,000 × *g* in a cooled microcentrifuge. The supernatant was either used immediately or frozen in liquid nitrogen and stored at −70 °C.

Immunoprecipitations and Western Blots—For isolation of FLAG-tagged proteins and associated proteins, Sf9 cell extracts were subjected to affinity purification on anti-FLAG beads (Sigma) as recommended by the manufacturer. Briefly, cell extracts were incubated with anti-FLAG beads at 4 °C with gentle agitation for 30 min. The resin was washed four times with Buffer A, and protein was eluted by incubation at 4 °C for 45 min with FLAG peptide (100 μg/ml) in Buffer B (20 mM Tris-HCl, pH 7.5, 70 mM KCl, 1 mM dithiothreitol, 2 mM $Mg(OAc)_2$, 10% glycerol). A fraction of the recovered proteins was subjected to SDS-PAGE and the gel was analyzed either by Coomassie Blue staining, exposure to x-ray film overnight to detect radioactive bands, or transfer of proteins to a polyvinylidene difluoride membrane (Millipore) for Western blotting. eIF3 subunits were detected with polyclonal goat anti-eIF3 antiserum (1:2000), whereas eIF3c-Myc was probed with monoclonal anti-Myc antibodies (1:2000, Santa Cruz Biotechnology). Protein bands were revealed by incubation with the appropriate alkaline phosphatase-conjugated secondary antibody.

Purification of eIF3 and 40 S Ribosomal Subunits—eIF3 was purified from HeLa cells as described previously (23), with some modifications. Briefly, HeLa cell lysate from 200 g of cell pellet was passed through Q Sepharose Fast Flow ion exchange media and eluted using a potassium chloride gradient. Fractions containing eIF3 were precipitated using ammonium sulfate and then passed through a Superdex 200 gel filtration column. Fractions were then diluted and purified using cation exchange. The purity of eIF3 was determined by SDS-PAGE and Coomassie Blue staining.

Ribosomal subunits were isolated from HeLa cells as described (24). Purity of the ribosomal subunits was assessed by sucrose gradient centrifugation; quality was demonstrated by their efficient formation of 80 S ribosomes in 5 mM $Mg(OAc)_2$ buffer (10, 24).

Assembly and Analysis of 40 S Ribosomal Complexes—40 S complexes were assembled by incubating purified 40 S ribosomal subunits (17 pmol) with either purified HeLa eIF3 (17 pmol), radiolabeled recombinant subcomplexes, or individual recombinant subunits isolated, and purified from insect cells. Following incubation for 3 min at 37 °C in Buffer B lacking glycerol, the reactions were chilled for 5 min on ice, layered over 10–40% (w/v) linear sucrose gradients containing buffer B, and centrifuged in a Beckman SW-40 rotor at 38,000 rpm for 3.5 h at 4 °C. After centrifugation, each gradient was fractionated using an ISCO gradient fractionator, and the absorbance profile at 254 nm was monitored. Fractions were collected, precipitated with methanol, and the presence of eIF3 subunits determined by SDS-PAGE and autoradiography and/or Coomassie Blue staining. Alternatively, the total radioactivity in each gradient fraction was determined by measuring trichloroacetic acid-precipitable radioactivity in a scintillation counter.

RESULTS

Expression of Human eIF3 Subunits in Sf9 Cells and Purification of the Recombinant Proteins—Construction and production of baculoviruses expressing each subunit of human eIF3 that has a homolog in *S. cerevisiae* (subunits a, b, c, g, i, and j) were performed as described under "Experimental Procedures." Initially, all but the eIF3c subunit was tagged at the N terminus with a FLAG peptide, allowing for more efficient purification. Extracts prepared from Sf9 cells infected with individual recombinant baculovirus strains were subjected to affinity purification by using anti-FLAG beads and proteins were eluted with the FLAG peptide as described under "Experimental Procedures." Each of the purified FLAG-tagged eIF3b, eIF3g, eIF3i, and eIF3j preparations exhibits a single major protein band, indicating that these proteins are stable as isolated subunits in insect cells (Fig. 1*A*). Each of the proteins has an apparent molecular weight equal or very close to the corresponding subunit derived from eIF3 purified from HeLa cells.

eIF3a also was expressed in Sf9 cells but does not accumulate to a high level in the soluble fraction of cell extracts (Fig. 1*B*, *lane 1*). Instead, a large amount of eIF3a was found in the insoluble fraction (data not shown), which may be due to denaturation or an association of eIF3a with components of the cytoskeleton (25–27). Therefore, we asked if coinfection of cells with other subunits of eIF3 might promote the solubility of eIF3a in cell extracts. Previously, both eIF3b and eIF3c have been shown to bind to eIF3a in mammalian (28) and yeast (29) eIF3. While eIF3c did not affect the solubility of eIF3a in Sf9 cells (data not shown), when cells were coinfected with viruses expressing eIF3a and eIF3b (Fig. 1*B*, *lane 2*) a significant amount of eIF3a became soluble in these cell extracts. This presumably reflects an association of the two proteins *in vivo*.

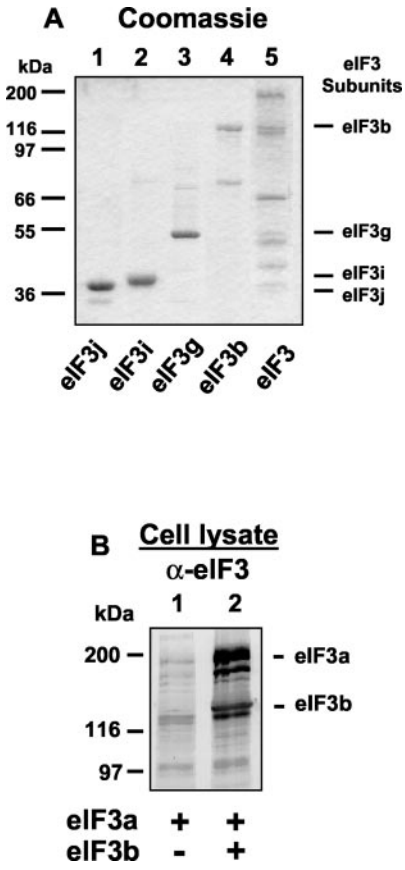

FIG. 1. **Overexpression of eIF3 subunits in baculovirus-infected Sf9 cells.** *A*, extracts prepared from Sf9 cells infected with the indicated baculoviruses were treated with anti-FLAG affinity beads, and proteins were eluted with FLAG peptide as described under "Experimental Procedures." The eluted proteins were resolved by 9% SDS-PAGE and stained with Coomassie Blue. Purified HeLa eIF3 (8 μg) was resolved as indicated (*lane 5*). *B*, Sf9 cells were infected with baculoviruses expressing eIF3a in the absence (*lane 1*) or presence (*lane 2*) of eIF3b virus, as indicated below each lane. Aliquots of extracts containing equal amounts of protein were resolved directly by SDS-PAGE, followed by immunoblotting with anti-eIF3 polyclonal antibody. The presence of eIF3a and eIF3b are indicated.

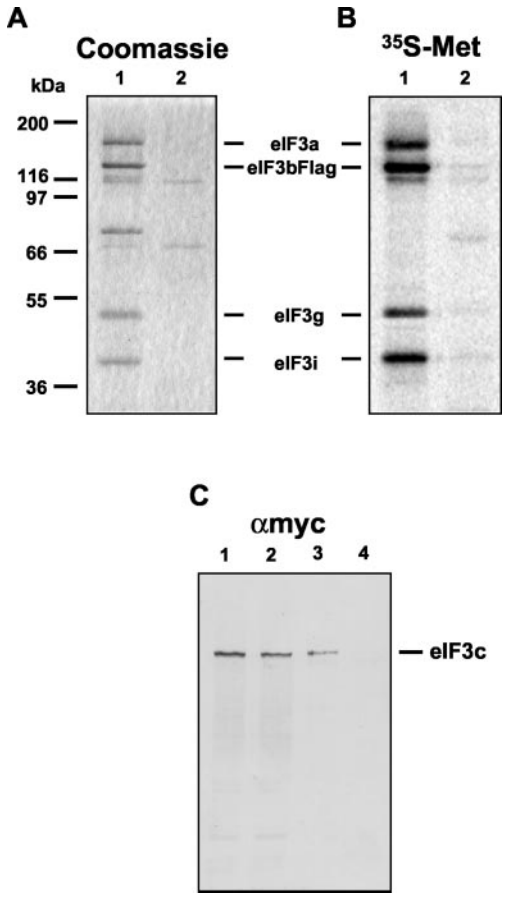

FIG. 2. **eIF3c associates weakly with a stable eIF3 core complex.** Sf9 cells were coinfected with baculovirus strains expressing eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i and were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection. *A*, cells were infected with viruses encoding either eIF3b tagged with a FLAG peptide linked to a heart muscle kinase phosphorylation peptide (HMK) (eIF3bFlag, *lane 1*), or untagged eIF3b (*lane 2*), together with a His_6-Myc-tagged eIF3c and untagged eIF3a, eIF3g, and eIF3i subunits. eIF3b and associated proteins were recovered using anti-FLAG affinity beads, and the recovered proteins were eluted and resolved by SDS-PAGE. The Coomassie Blue-stained gel is shown. *B*, autoradiograph of the gel shown in *A* is presented. *C*, experiment was repeated, but recovered proteins were resolved by SDS-PAGE and immunoblotted with anti-Myc antiserum to identify eIF3c in the FLAG-tagged complex. *Lanes 3* and *4* show the recovered proteins following elution from anti-FLAG beads in the presence of FLAG-HMK eIF3b (*lane 3*) and in the presence of untagged eIF3b (*lane 4*). *Lanes 1* and *2* show 1% of the amounts of cell extract used for the affinity purifications shown in *lanes 3* and *4*, respectively. The migration position of eIF3c is indicated.

Our results show for the first time that the baculovirus overexpression system efficiently produces relatively large amounts of eIF3 subunits that generally are not proteolyzed, in contrast to what is seen when *Escherichia coli* is used to express the larger subunits (subunits a, b, and c).[2] However, the expression of eIF3b in Sf9 cells does result in a minor amount of this protein being cleaved into at least two distinct fragments. The N-terminal fragment (~70 kDa) is recovered on anti-FLAG beads by virtue of its FLAG tag, and is readily detected by Coomassie Blue staining (Fig. 1*A*). However, the N-terminal fragment does not label efficiently with [^{35}S]methionine (Fig. 2*B*), as it possesses only 3 of 16 methionines found in eIF3b. The extent of cleavage of eIF3b during its overexpression in Sf9 cells was found to vary between experiments, although the reasons for this variability are not clear.

Complex Formation and Purification of Human eIF3 Subcomplexes—Following the observation that human eIF3 subunits are efficiently expressed in Sf9 cells using the baculovirus expression system, we sought to determine whether these subunits would form a stable subcomplex that resembles the *S. cerevisiae* eIF3 core complex. To this end, we coinfected Sf9 cells with 5 different recombinant baculovirus strains encoding the a, b, c, g, and i subunits of human eIF3. To allow for rapid purification of the resulting complex, eIF3b was expressed as a FLAG-tagged fusion protein, while the other subunits were expressed as untagged proteins. Efforts were made to adjust the amounts of viruses used during the coinfection so that each subunit would be expressed in approximately stoichiometric amounts. Purification with anti-FLAG beads results in a complex that possesses at least four of the eIF3 subunits (eIF3a, eIF3b, eIF3g, and eIF3i), as observed by Coomassie Blue staining (Fig. 2*A*, *lane 1*) and autoradiography (Fig. 2*B*, *lane 1*). As a negative control, a similar coinfection was performed with untagged eIF3b; none of the eIF3 subunits binds to the FLAG beads (Fig. 2, *A* and *B*, *lane 2*). The finding that three subunits co-elute with FLAG-eIF3b in approximately stoichiometric amounts suggests that all four proteins are present together in a complex (but see "Discussion" below).

Since eIF3b and eIF3c do not separate well by SDS-PAGE, it was important to determine whether eIF3c also associates with the complex. To answer this question, a His_6-Myc-tagged eIF3c

[2] H-P. Vornlocher and K. Block, unpublished results.

subunit was used during coinfections as described above. Immunoblotting with specific anti-Myc antibodies indicates that the cell extract contains soluble eIF3c prior to FLAG purification (Fig. 2*C*, *lanes 1* and *2*). However, only a very small amount (~0.5%) of available eIF3c associates with the purified FLAG-tagged eIF3 subcomplex (Fig. 2*C*, *lane 3*), and no eIF3c is found in the control purification lacking the FLAG tag (Fig 2*C*, *lane 4*). In addition, immunoprecipitation of this particular coinfection with an anti-Myc affinity resin shows purification of His_6-Myc-eIF3c without appreciable amounts of the other eIF3 subunits (data not shown). Therefore, eIF3c is able to associate with the four-subunit eIF3 subcomplex, but does so only weakly under these expression and purification conditions. We conclude that the tags on eIF3c are not the cause of its inability to bind stably, because untagged eIF3c also does not incorporate efficiently into the eIF3abgi subcomplex (data not shown). Similarly, the tag on eIF3b likely does not impede eIF3c incorporation into the complex, because the complex purified through a His_6-tagged eIF3a instead of FLAG-eIF3b also is deficient in eIF3c (data not shown).

Interactions between the Human eIF3 Core Subunits—Since the physical linkages among human eIF3 subunits have not been fully elucidated, we investigated subunit interactions in the eIF3 subcomplexes using the baculovirus expression system. By coexpressing combinations of the four eIF3 subunits in Sf9 cells and isolating complexes using a single FLAG-tagged subunit in each case, we have verified that the interactions between the human subunits resemble those in yeast eIF3 (29, 30). Subcomplexes were isolated that contain b-g, b-i, b-j, b-g-i, and b-i-j (Fig. 3). eIF3b appears to be a central scaffolding subunit to which most, if not all, of the other eIF3 core subunits bind, including eIF3a, eIF3g, eIF3i, and eIF3j (Figs. 1 and 3, and data not shown). Unfortunately, the low affinity of eIF3c to the four-subunit subcomplex in this system prevents us from determining its protein interactions at the present time. Interestingly, the binding of either eIF3g or eIF3i to eIF3b is enhanced by the presence of the other subunit (Fig. 3). Additionally, eIF3g and eIF3i are able to form a stable dimer, but do not bind individually or as a dimer to eIF3j (Fig. 3*A*, *lane 5* and data not shown). These types of mapping experiments suggest that human and yeast eIF3 have similar core structures.

eIF3j Promotes the Stable Association of eIF3 Subcomplexes to the 40 S Ribosomal Subunit—In light of the above observations, we asked whether or not a subcomplex of human eIF3 composed of the eIF3a, eIF3b, eIF3g, and eIF3i subunits (eIF3abgi) is sufficient to confer the ribosome binding function of eIF3. This seemed rather likely, as all the subunits have been identified as RNA-binding proteins except eIF3i, and purified mammalian eIF3 binds *in vitro* to 40 S ribosomal subunits with high affinity (Ref. 1). and data not shown). To test for 40 S ribosome binding, we purified the four-subunit subcomplex containing FLAG-tagged eIF3b that had been labeled with [^{35}S]methionine (Fig. 4*A*, *lane 2*). Note that in this preparation, the eIF3a subunit is substoichiometric. *In vitro* binding was tested by incubating the radiolabeled subcomplex with 40 S ribosomal subunits and analyzing the mixture by sucrose gradient centrifugation (Fig. 4*B*, *lower panel*). Surprisingly, the four-subunit subcomplex does not stably associate with 40 S ribosomal subunits, although radioactivity between the top of the gradient and the 40 S peak indicates a weak interaction (Fig. 4*B*, *lower panel*). We then asked if the subcomplex required additional eIF3 subunits in order to bind 40 S ribosomes more tightly. A radiolabeled five-subunit eIF3abgij subcomplex was generated by coinfection and purification by using FLAG-tagged eIF3j while the other four subunits were untagged (Fig. 4*A*, *lane 1*). A stable association of the subcomplex with the 40 S ribosomal subunits is observed (Fig 4*B*, *upper panel*). Analysis of the bound eIF3 subunits by SDS-PAGE showed that all of the subunits except eIF3a are present on the 40 S ribosomal subunit (results not shown; see Fig. 7*A*). We conclude that eIF3j is needed to stabilize the binding of a subcomplex containing eIF3bgi. The role of eIF3a is unclear, as it appears to have been degraded during the experiment (results not shown). Purification of eIF3 from HeLa cells and rabbit reticulocytes often results in proteolysis of eIF3a (Refs. 28 and 31, and data not shown), so it is possible that the eIF3a subunit in our subcomplexes may be even less stable than eIF3a present in eIF3 prepared from whole cells.

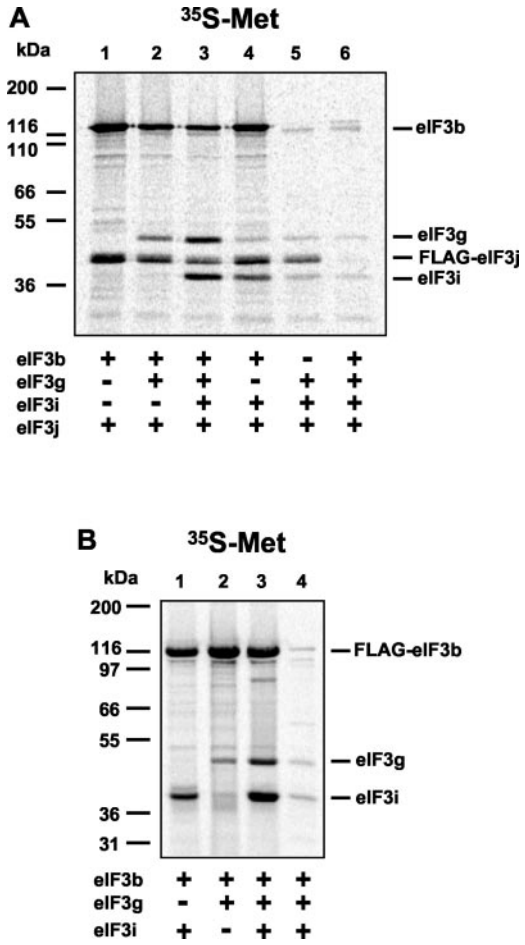

FIG. 3. **Interactions of human eIF3 subunits.** *A*, Sf9 cells were infected with combinations of baculovirus strains encoding eIF3b, eIF3g, and eIF3i, together with either FLAG-HMK-tagged eIF3j (*lanes 1–5*) or untagged eIF3j (*lane 6*), as indicated below each lane. Cells were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection. Extracts were prepared and treated with anti-FLAG beads. Recovered proteins were eluted from the beads using FLAG peptide and were resolved by SDS-PAGE. The resulting autoradiograph of the gel is shown, and the bands corresponding to eIF3b, eIF3g, eIF3i, and FLAG-eIF3j are indicated. *B*, Sf9 cells were infected with baculovirus strains expressing either FLAG-tagged eIF3b (*lanes 1–3*) or untagged eIF3b (*lane 4*), together with eIF3g (*lane 2*), eIF3i (*lane 1*), or both eIF3g and eIF3i (*lanes 3* and *4*). Cells were grown in the presence [^{35}S]methionine between 24 and 60 h post-infection, and FLAG-tagged eIF3b and associated proteins were recovered and analyzed as described in *A*. An autoradiograph of the gel is shown.

eIF3j Binds Specifically to the 40 S Ribosomal Subunit in Vitro—The above results suggest that eIF3j may bind to the 40 S ribosomal subunit directly, or that its presence enhances the affinity of other eIF3 subunit(s) to the 40 S ribosomal subunit. To investigate whether eIF3j alone can bind to the 40 S ribosomal subunit, we purified radiolabeled FLAG-eIF3j and incubated it with isolated 40 S ribosomal subunits. Ribosomal bind-

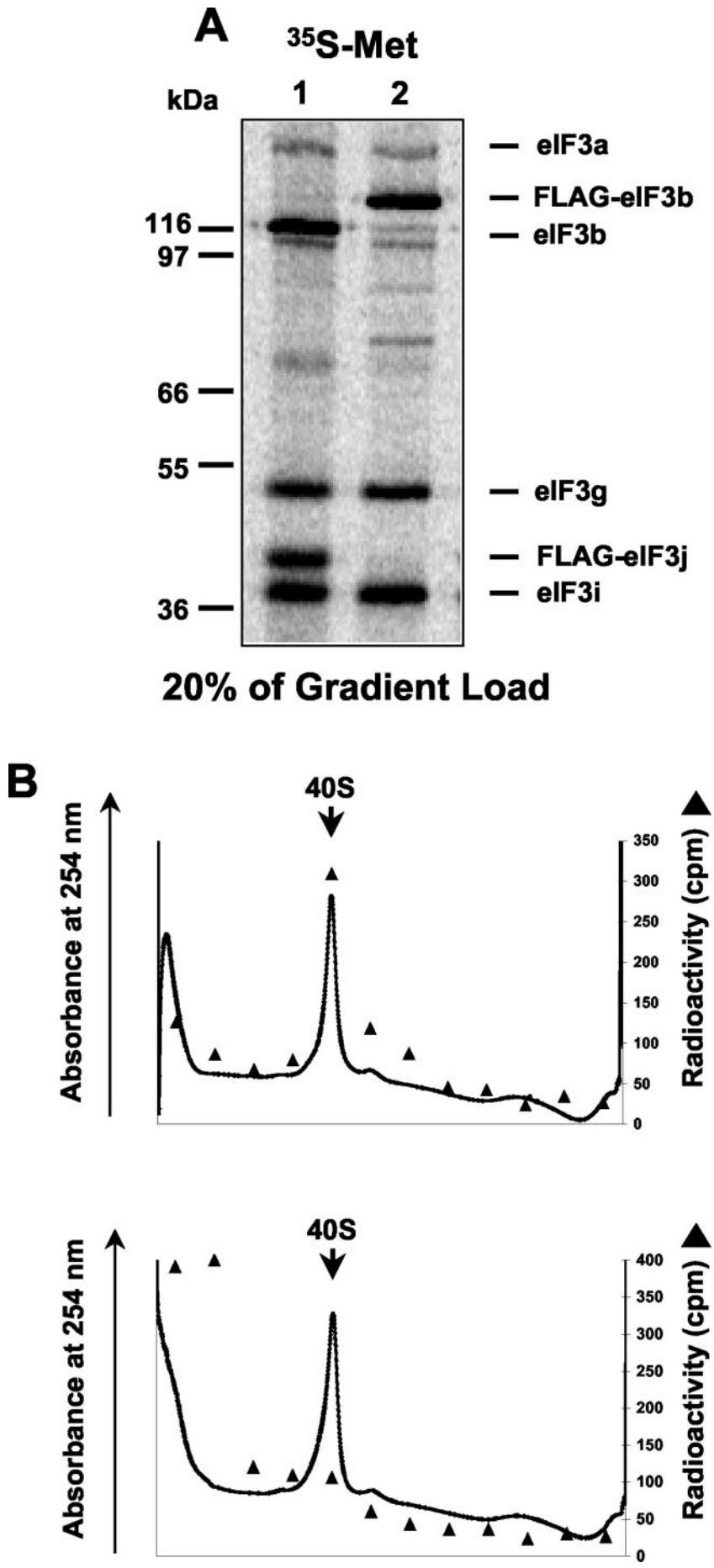

FIG. 4. **eIF3j stabilizes eIF3bgi binding to 40 S ribosomal subunits *in vitro*.** *A*, two eIF3 subcomplexes were produced in Sf9 cells as follows. Cells were infected with baculovirus strains encoding untagged eIF3a, eIF3b, eIF3i, and eIF3g, and FLAG-HMK-eIF3j (*lane 1*) and untagged eIF3a, eIF3i and eIF3g, and FLAG-HMK-eIF3b (*lane 2*). Cells were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection, harvested and treated with anti-FLAG beads. Proteins were eluted from the beads as described in the legend to Fig. 3, and proteins (20%) were resolved by SDS-PAGE and visualized by autoradiography. *B*, remainder of the affinity-purified proteins (80%) was analyzed for binding to 40 S ribosomal subunits as described under "Experimental Procedures." Gradient fractions were precipitated with trichloroacetic acid, and radioactivity was counted and plotted with the corresponding absorbance profiles (254 nm). Sedimentation is from left to right. The *upper panel* corresponds to the subcomplex purified in *lane 1* of *panel A*, whereas the *lower panel* corresponds to *lane 2* of *panel A*.

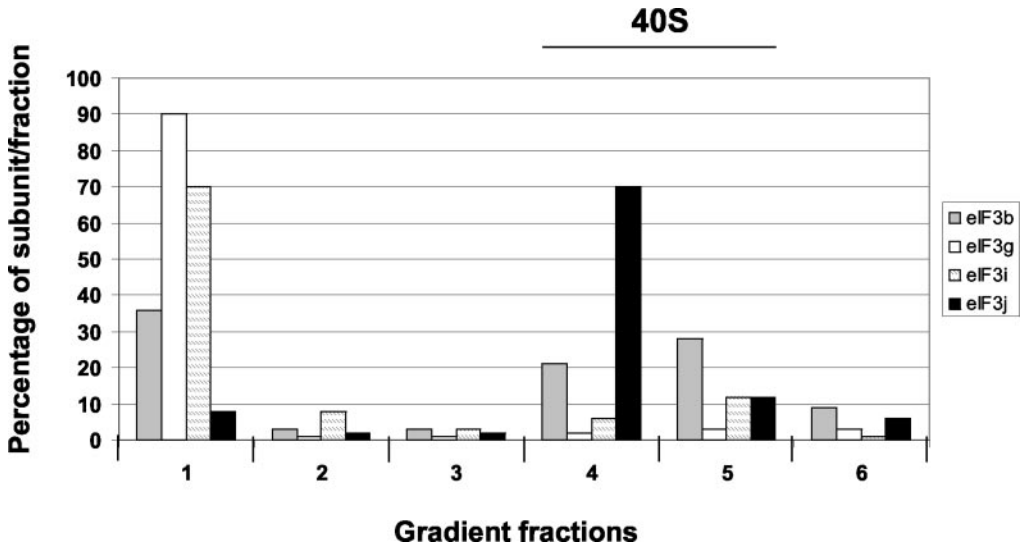

FIG. 5. **eIF3b and eIF3j individually bind to the 40 S ribosomal subunit *in vitro*.** Sf9 cells were individually infected with baculovirus strains encoding eIF3j, eIF3i, eIF3g, and eIF3b, all FLAG-tagged. The cells were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection and the FLAG-tagged proteins were purified with an anti-FLAG affinity resin. The recovered proteins were then analyzed for binding to 40 S ribosomal subunits as described under "Experimental Procedures" and the legend to Fig. 4*B*. The radioactivity in each fraction is reported as the percent of total radioactivity for that subunit. The location of the 40 S ribosomal subunits is indicated above the bar graph; fractions are numbered from the top of the gradient.

ing was determined by sucrose gradient centrifugation, where the 40 S subunits are found in fractions 4 and 5 (Fig. 5). Most of the eIF3j is found in fraction 4, indicating that the subunit does, in fact, bind stably to the 40 S ribosomal subunit in the absence of other translational components. We also tested whether FLAG-tagged eIF3b, eIF3g, and eIF3i individually bind to the 40 S ribosomal subunit, even though as a complex the three subunits do not. eIF3b independently associates with modest affinity to the 40 S ribosomal subunit, whereas eIF3g and eIF3i do not bind at all (Fig. 5). Since eIF3b alone binds more tightly than the eIF3bgi subcomplex, we suspect that the binding seen with eIF3b is artifactual and may reflect its incorrect protein folding in the absence of binding partners.

Having demonstrated that eIF3j possesses 40 S ribosomal subunit affinity, both alone and in eIF3 subcomplexes, we wished to demonstrate that the binding occurs specifically at a single site on the 40 S subunit. 40 S binding was tested with increasing amounts of eIF3j in an effort to saturate the putative 40 S ribosome binding site. Radiolabeled FLAG-tagged eIF3j was expressed in Sf9 cells and purified by using the anti-FLAG affinity resin. The concentration of purified eIF3j was determined by Coomassie Blue staining and different amounts of eIF3j were incubated with isolated 40 S ribosomal subunits (Fig. 6, *panels A–E*). When amounts of eIF3j less than or equal to the amount of 40 S ribosomes are tested, all of the labeled subunit is found in the 40 S region of the gradient, indicating very tight binding. When the amount of eIF3j is about twice the amount of 40 S subunits, about half of the eIF3j is bound, whereas about half is present at the top of the gradient (Fig. 6*E*). Saturation is reached when about 19 pmol of eIF3j are added to 17 pmol of 40 S ribosomal subunits. This is consistent with a stoichiometry of a single molecule of eIF3j binding to a 40 S ribosomal subunit. Further evidence that this binding is specific comes from the finding that eIF3j does not bind to purified 60 S ribosomal subunits (Fig. 6*F*).

The Subcomplex eIF3bgij Binds to the Correct Site on the 40 S Ribosomal Subunit—Since eIF3j binds to the 40 S ribosomal subunit directly, we wished to obtain evidence that the eIF3j-stabilized subcomplex, eIF3bgij, binds to the same site as native eIF3. It is well established that purified mammalian eIF3 is able to promote the formation of a stable 40 S preinitiation complex (1–3, 31). We therefore asked whether or not purified human eIF3 is able to compete with the recombinant eIF3 subcomplex for 40 S binding. If the subcomplex binds to the correct site on the 40 S ribosomal subunit, prior incubation of 40S subunits with purified human eIF3 should reduce the association of recombinant eIF3bgij with the 40 S ribosomal subunit. To this end, radiolabeled recombinant eIF3 subcomplex (eIF3bgi with FLAG-eIF3j) was expressed and purified on an anti-FLAG affinity resin as described under "Experimental Procedures." The subcomplex was incubated with purified 40 S subunits or with 40 S subunits that had been preincubated with purified human eIF3, and binding to the 40 S subunit was monitored by sucrose gradient centrifugation and SDS-PAGE (Fig. 7). The presence of the radiolabeled subcomplex was detected by autoradiography and human eIF3 subunits were identified by Western blotting (data not shown). As shown in Fig. 7, the recombinant eIF3 subcomplex is found predominantly (75%) in the 40 S region of the gradient (*panel A*). However, preincubation of the 40 S ribosomal subunits with purified human eIF3 reduces the subsequent association of the subcomplex with 40 S ribosomal subunits, with only 30% of the subcomplex in the 40 S region (*panel B*). The ability of purified human eIF3 to compete for binding to the 40 S ribosomal subunit was found to be dose-dependent (data not shown). Additionally, prior incubation of 40 S subunits with purified eIF3 prevents eIF3j from associating with the 40 S ribosomal subunits (data not shown). These results indicate that the 40 S binding site for recombinant eIF3bgij overlaps the eIF3 binding site, and suggest that the subcomplex binds at the correct eIF3 site on 40 S ribosomal subunits.

Since eIF3j binds directly to the 40 S ribosomal subunit *in vitro*, it is possible that such binding would block the binding of native eIF3 *in vivo*. We overexpressed FLAG-eIF3j in HEK 293T cells to analyze 40 S ribosomal subunit binding in its native cellular milieu. Following transfection, a portion (less than 10%) of the overexpressed FLAG-eIF3j was found bound to 40 S subunits in these cells (data not shown). Polysome profiles and protein synthesis rates were not affected by the overexpression of eIF3j (data not shown). This indicates that eIF3j binding to 40 S ribosomal subunits *in vivo* does not compete with endogenous eIF3 for 40 S ribosomal binding. This

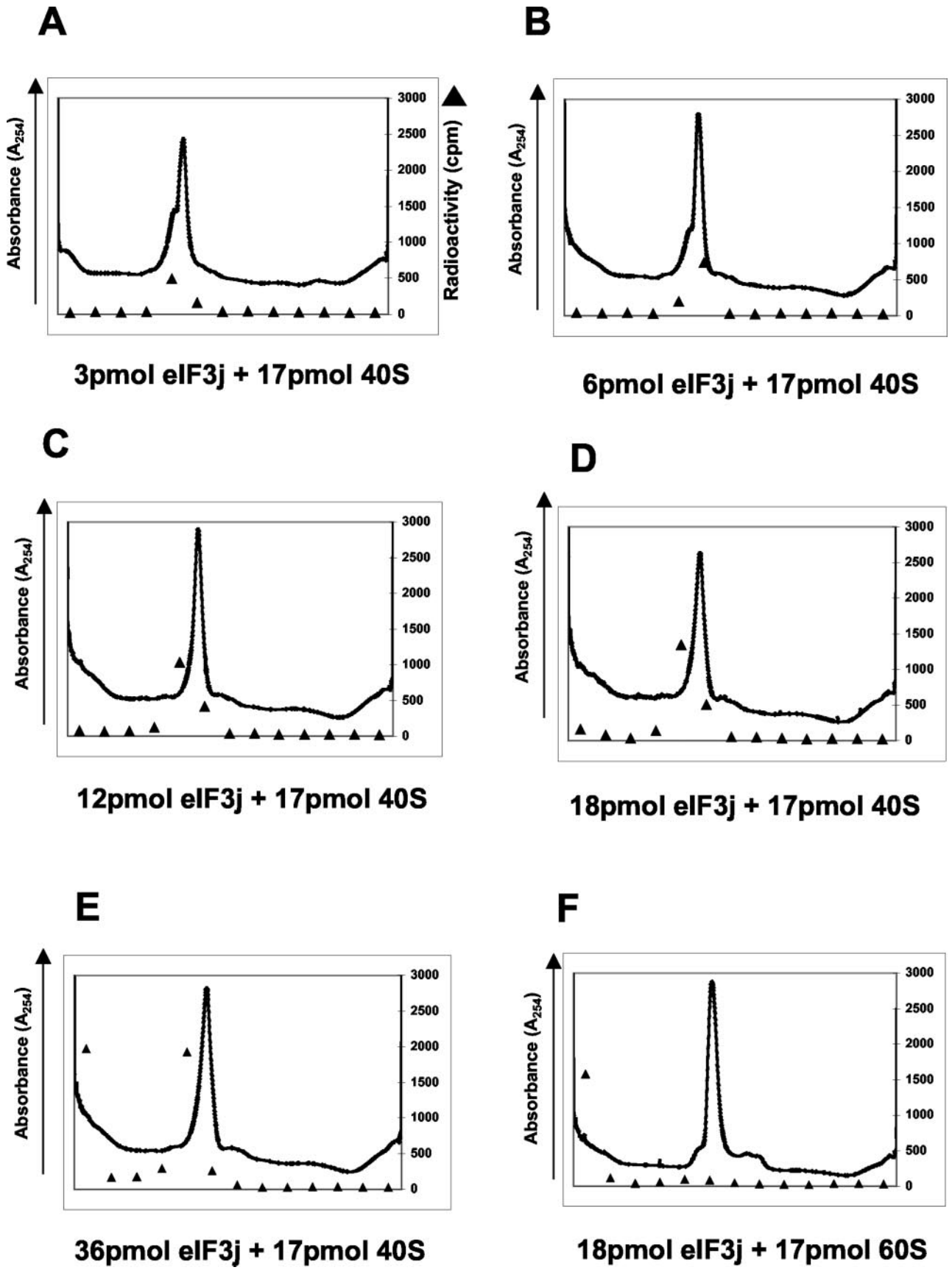

FIG. 6. **eIF3j binds stoichiometrically to the 40 S ribosomal subunit *in vitro*.** Sf9 cells were infected with baculoviruses encoding FLAG-tagged eIF3j. The cells were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection and the FLAG-tagged protein was purified using anti-FLAG beads. The protein was eluted with FLAG peptide and the concentration of the recovered protein was determined with bovine serum albumin as standard. The indicated amounts of eIF3j were assayed for binding to 40 S (*panels A–E*) or 60 S (*panel F*) ribosomal subunits as described under "Experimental Procedures" and the legend to Fig. 4*B*. Sedimentation is from left to right; radioactivity (cpm) is plotted with the absorbance profile.

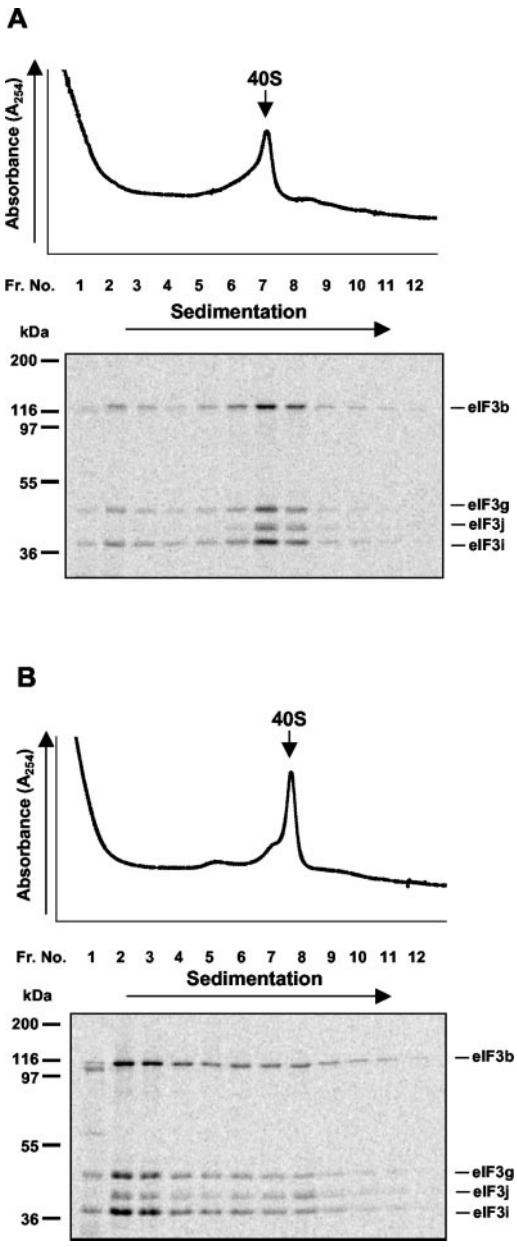

FIG. 7. **The eIF3bgij subcomplex competes with purified eIF3 for 40 S ribosomal binding *in vitro*.** Sf9 cells were coinfected with baculovirus strains encoding untagged eIF3b, eIF3i, and eIF3g and FLAG-HMK-eIF3j. The cells were grown in the presence of [^{35}S]methionine between 24 and 60 h post-infection and lysate proteins were treated with anti-FLAG affinity resin. The eluted proteins were assayed for binding to 40 S ribosomal subunits as described under "Experimental Procedures," except that the ribosomes had been preincubated in the absence (*A*) or presence (*B*) of purified eIF3 (20 pmol) for 3 min at 37 °C. Following centrifugation, gradient fractions were treated with methanol and the precipitated proteins were resolved by SDS-PAGE. The resulting gel autoradiographs are aligned under the gradient absorbance profiles.

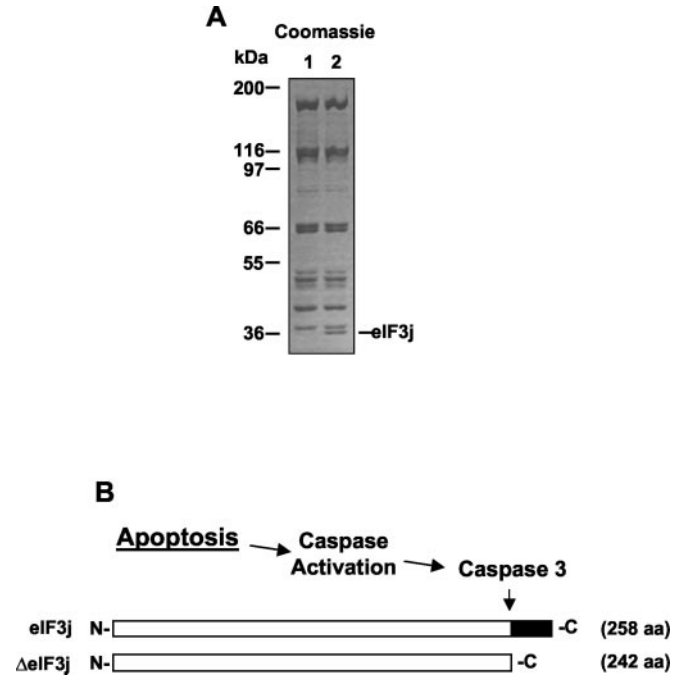

FIG. 8. **Purification of HeLa cell eIF3.** *A*, HeLa cell eIF3 was purified as described under "Experimental Procedures." Following ion exchange chromatography, the quality of purified eIF3 was determined by SDS-PAGE and Coomassie staining. It was possible to separate eIF3 deficient in eIF3j (*lane 1*) from the complex that contains eIF3j (*lane 2*). *B*, caspase-3 directly cleaves eIF3j, resulting in the loss of 16 amino acids from its C terminus (32). A schematic representation of the cleavage of eIF3j during apoptosis is shown.

could be explained by the ready dissociation of eIF3j from eIF3, allowing eIF3 lacking the j-subunit to bind to 40 S ribosomes already saturated with the overexpressed eIF3j.

eIF3j Is Required for the Stable 40 S Binding of eIF3 in Vitro—The stabilization of eIF3bgi binding to 40 S ribosomal subunits by eIF3j suggests that this subunit may play an important role in the binding of the entire eIF3 complex. During our routine purification of eIF3 from HeLa cells (see "Experimental Procedures"), two forms of eIF3 were obtained; one possesses all 12 subunits, whereas the other lacks only the eIF3j subunit (Fig. 8*A*). The complete 12-subunit eIF3 complex binds tightly to 40 S ribosomal subunits (results not shown), but the eIF3 lacking eIF3j binds very poorly (Fig. 9*A*). eIF3 subunits are hardly detected at all in the 40 S region of the gradient, but are present near the top in fractions 3 and 4, as expected for a complex of about 15 S. In striking contrast, when eIF3j is added to the complex lacking this subunit, and is tested for binding to 40 S ribosomal subunits, tight binding of eIF3 is restored (Fig. 9*B*). In this case, about 70% of the eIF3 is found in the 40 S region of the sucrose gradient, and little evidence of trailing toward the top is seen. A lack of trailing indicates that the binding is quite stable, and that little eIF3 is released from the 40 S particles during the 3.5 h of centrifugation. The immunoblot analysis of eIF3j (Fig. 9*B*, *lower panel*) shows that the subunit is present in the eIF3–40S complex. We also have determined that FLAG-eIF3j incorporates efficiently into the eIF3j-deficient eIF3 complex in the absence of ribosomes (data not shown). The results indicate that eIF3j is required for stable eIF3 binding to 40 S ribosomes, at least in this *in vitro* assay system.

Cleavage of eIF3j by Caspase-3 Reduces the Affinity of eIF3 for the 40 S Ribosomal Subunit in Vitro—Previous work has identified eIF3j as a target of cleavage by caspase-3 during apoptosis *in vivo* (32). eIF3j is predominantly cleaved between residues 242 and 243, resulting in a C-terminal truncated protein lacking 16 amino acid residues (Fig. 8*B*, ΔeIF3j). We hypothesize that this cleavage event may alter the affinity of eIF3j and perhaps eIF3 for 40 S ribosomal subunits. Indeed, FLAG-ΔeIF3j alone binds poorly to 40 S ribosomal subunits (data not shown), whereas the uncleaved full-length subunit binds well (Fig. 5). Thus, the loss of the C-terminal peptide substantially reduces the protein binding affinity with the 40 S ribosome. To test the effect of eIF3j cleavage on eIF3 binding, we made use of the purified eIF3 that lacks eIF3j described above (Fig. 8*A*). First, we determined that FLAG-ΔeIF3j is

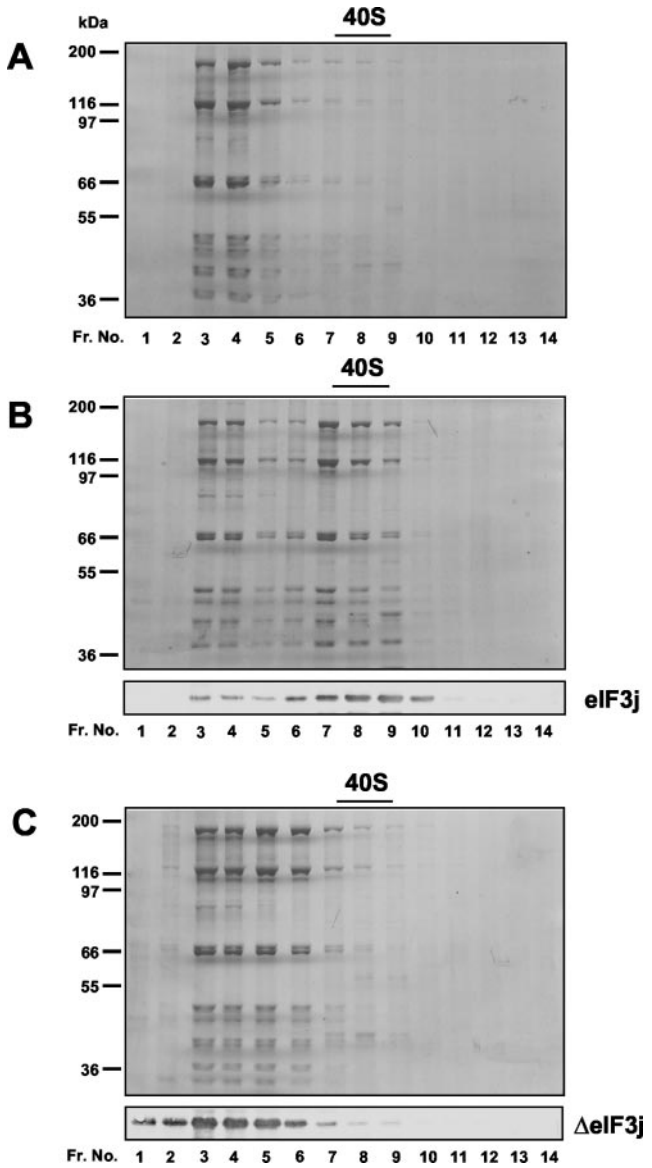

FIG. 9. **The j-subunit of eIF3 is required for the stable binding of the eIF3 complex to the 40 S ribosomal subunit *in vitro*.** *A*, HeLa cell eIF3 that is deficient in eIF3j (25 pmol) was analyzed for binding to 40 S ribosomal subunits as described under "Experimental Procedures." Following fractionation of the gradients, each fraction was precipitated with methanol, and the presence of eIF3 was determined by SDS-PAGE and Coomassie staining. *B*, HeLa cell eIF3 deficient in eIF3j (25 pmol) was incubated with purified FLAG-eIF3j (25 pmol) and then analyzed for binding to 40 S ribosomal subunits as described for *panel A* (*upper panel*). The presence of FLAG-eIF3j was determined by SDS-PAGE and immunoblotting with anti-eIF3 polyclonal antibody (*lower panel*). *C*, HeLa cell eIF3 deficient in eIF3j (25 pmol) was incubated with purified FLAG-ΔeIF3j (25 pmol) and binding to the 40 S ribosomal subunit was determined as described in *panel A* (*upper panel*). The presence of FLAG-ΔeIF3j in each fraction was visualized by immunoblotting with anti-eIF3 polyclonal antibody (*lower panel*). The results presented are from a single experiment but are representative of those obtained in three separate experiments.

stably incorporated into the eIF3j-deficient eIF3 complex (data not shown), suggesting that the C terminus is not involved in eIF3j binding to the eIF3 complex. Addition of FLAG-ΔeIF3j to eIF3j-deficient eIF3 leads to only a modest stabilization of eIF3 subunits on the 40 S ribosome (Fig. 9*C*). In this case, fewer than 5% of the eIF3 subunits are present in the 40 S region of the sucrose gradient compared with 70% when full-length eIF3j is added (Fig. 9*B*). However, there is considerable trailing of eIF3 subunits from the 40 S region toward the top of the gradient, indicating that FLAG-ΔeIF3j confers some stabilization of eIF3 binding. The results suggest that residues 243 to 258 in eIF3j are necessary for stable association of eIF3 with 40 S ribosomal subunits.

DISCUSSION

Since its initial characterization in the 1970s, the structure and function of the mammalian multisubunit initiation factor eIF3 remains poorly defined. The composition and stoichiometry of the subunits have not been determined rigorously, high resolution structures are unavailable, and the functions of individual subunits are yet to be elucidated. Recently, molecular genetic analyses have led to significant advances in our understanding of eIF3 in the budding yeast, *S. cerevisiae* (33). Yeast eIF3 is composed of five core subunits (eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i) and one substoichiometric component (eIF3j), compared with human eIF3, which contains at least 12 nonidentical subunits (5, 6). All five of the core subunits are required for growth of yeast (33). The purified five-subunit core complex restores the binding of Met-tRNA$_i$ and mRNA to 40 S ribosomes in heat-inactivated *prt1-1* (eIF3b) mutant extracts (17, 18, 34), suggesting that this core complex represents the minimal functional form of yeast eIF3. However, the addition of a purified stable eIF3abc subcomplex also rescues the mutant lysate, whereas addition of eIF3bgi does not (34), suggesting that eIF3abc may be the minimal active eIF3 complex. However, the experiments do not rule out the possibility that one or more other endogenous eIF3 subunits bind to eIF3abc (but not to eIF3bgi) to generate a larger complex with sufficient activity to rescue the lysate. In fact, the authors report a substantial proportion of eIF3j remains bound to the 40 S ribosome following dissociation of other factors on heat treatment of the *prt1-1* extract (34). It also should be noted that many of the yeast translation extract experiments employed formaldehyde fixation prior to centrifugal analysis (18, 34). As fixation with formaldehyde may complicate the interpretation of results, alternate approaches are needed to substantiate conclusions about yeast eIF3.

Studies of mammalian eIF3 are complicated by the overall structural complexity of the initiation factor and by the lack of facile genetic approaches. Ideally, we would like to be able to reconstitute eIF3 from its separated purified subunits. However, attempts to dissociate eIF3 subunits by mild denaturing agents such as urea led to insoluble proteins and a failure to reconstitute.[2] Expression of individual human eIF3 subunits in *E. coli* produces soluble, fairly stable proteins for subunits with masses below 70,000 kDa, but the three high molecular weight subunits, eIF3a, eIF3b, and eIF3c, are either insoluble or readily degraded. We therefore turned to the baculovirus system as a means to synthesize amounts of the human eIF3 subunits adequate for biochemical analysis. We first turned our attention to the five subunits (a, b, c, g, and i) that share homology with the components of the yeast core complex, with the intent to reconstitute a 5-subunit complex that could be purified and tested for eIF3 activity in a variety of *in vitro* assays. Co-infection of Sf9 cells with the five recombinant viruses allows the isolation in good yield of an eIF3 complex with four of the subunits, plus only a small amount of eIF3c even though the subunit accumulated as a soluble protein. The problem of efficient eIF3c incorporation into the eIF3abgi complex has yet to be solved; perhaps one of the other eIF3 subunits or some other protein is required for its incorporation. It is noted that purified eIF3 from *S. cerevisiae* also possesses substoichiometric amounts of eIF3c (35), so this subunit may be less tightly associated with the eIF3 complex than previously thought. Despite the problem with eIF3c, the baculovirus system appears ideally suited for constructing milligram amounts

of other subcomplexes with human eIF3 subunits, and such experiments are in progress.

The formation of the eIF3abgi and eIF3bgij subcomplexes in high yields indicates that the system also is highly suited to defining subunit-subunit interactions. The formation of dimeric complexes of a-b, b-g, b-i, b-j, and g-i has led to a model where eIF3b acts as a scaffold to which the other four subunits bind. The formation of stable dimeric complexes constitutes good evidence that the subunits involved interact directly in the eIF3 complex. The subunit interactions identified here also are seen in yeast eIF3 (33), based on results from the yeast two-hybrid system, GST pull-downs, Far-Western blotting, and genetic interactions. Whereas these methods have proved very effective in characterizing and mapping yeast eIF3 subunit interactions, they give unreliable results when applied to human eIF3[3]; most interactions are weak and many more interactions are detected than can readily be accommodated in a 3-dimensional model of eIF3. The problems encountered suggest that when a mammalian eIF3 subunit is isolated, it is capable of interacting non-specifically with other proteins also normally found in protein complexes. However, when two soluble proteins form a stable complex that can be purified, we suggest that this indicates a physiologically relevant interaction has formed.

The simplest measurable activity for eIF3 is its binding to purified 40 S ribosomal subunits in the absence of all other translational components (1). For an initial characterization of the activities of the eIF3 subcomplexes, this 40 S binding assay was used. We found that eIF3abgi and eIF3bgi do not bind stably to 40 S ribosomes when assayed by sucrose gradient centrifugation. However, when eIF3j is incorporated into the eIF3bgi complex, stable binding results. More importantly, eIF3 lacking eIF3j also binds poorly to 40S ribosomal subunits, but is greatly stabilized by the addition of eIF3j. The results indicate that eIF3j is the most important eIF3 subunit for forming a stable eIF3–40S complex, since the eIF3j-deficient eIF3 contains all of the other subunits yet its binding affinity is weak. This is the first case where a clear functional role for a mammalian eIF3 subunit is defined. In yeast, it has been reported that both eIF3a and eIF3j are required for binding of the eIF3bgi subcomplex to 40 S ribosomes *in vivo* (19). Additionally, yeast eIF3j binds to 18 S ribosomal RNA in an *in vitro* Northwestern analysis (20), yet is not essential for cell growth (36). The results from yeast are in partial agreement with data presented here. It is suggested that yeast eIF3a plays the dominant role in eIF3–40S binding, whereas our work with the human factor emphasizes the role of eIF3j. The differing conclusions concerning the importance of eIF3a and eIF3j likely are due to different methods used for testing the activity of eIF3–40S binding in the two systems.

Given the important role we have assigned to human eIF3j, it is surprising that eIF3j in *S. cerevisiae* is not an essential protein (36). However, the 40 S ribosomal subunit deficiency and hypersensitivity to paromomycin of yeast cells lacking eIF3j suggest that the subunit is required for optimal protein synthesis. It has been proposed that yeast eIF3j is a loosely associated, substoichiometric subunit of eIF3, which stabilizes a complex of initiation factors (eIF3, eIF5, eIF1, and eIF2) and possibly has a role in the biogenesis of 40 S ribosomal subunits (20, 29). The easy dissociation of eIF3j from eIF3, seen in both yeast and mammalian cells, suggests that eIF3j may exist both in and out of the eIF3 complex. Interestingly, purified yeast eIF3 that lacks the eIF3j subunit has been shown to function in stabilization of Met-tRNA$_i$ (17, 18) and mRNA (34) binding to 40 S ribosomes in heat-inactivated mutant *prt1-1* (eIF3b) extracts. However, it has not been ruled out that endogenous eIF3j associates with the purified eIF3 core complex to stimulate the activity of the *prt1-1* extract above. In contrast, a preparation of yeast eIF3 that lacks eIF3j does not function in a yeast translation initiation system reconstituted with purified components (35). Since our data suggest that human eIF3 requires eIF3j for stable association to 40 S ribosomal subunits *in vitro*, an eIF3 complex lacking this subunit may well function less efficiently in a translation initiation system reconstituted with purified components.

The induction of apoptosis results in the cleavage by caspase-3 of a number of initiation factors, including eIF3j (32, 37–39). These observations led us to speculate that caspase-3 cleavage of eIF3j between residues 242 and 243 alters its affinity for 40S ribosomal subunits or its incorporation into eIF3. eIF3j binding to purified 40 S ribosomes indeed is reduced *in vitro* when recombinant eIF3j is cleaved with caspase-3 (data not shown), or when a truncated mutant form is employed. However, the cleavage event does not prevent eIF3j from being incorporated into a subcomplex with eIF3bgi, or its association with purified eIF3. This result agrees with previous work indicating that cleaved eIF3j resides in a complex containing eIF4F and presumably eIF3 (32). To investigate whether the same 40 S binding behavior is observed *in vivo*, transiently transfected HEK 293T cells overexpressing HA-tagged ΔeIF3j were subjected to sucrose gradient analysis. Overexpressed HA-ΔeIF3j was present (~10%) in the 40 S region of the gradient and at the top, but not in between (data not shown), suggesting stable saturation binding. That ΔeIF3j is still able to associate with the 40 S ribosome under these conditions suggests that the cleavage of eIF3j by caspase-3 as a single event does not prevent its association with the 40 S ribosomal subunit. Apparently other initiation factors that interact with eIF3 are able to enhance the affinity of the eIF3 complex for 40 S ribosomes *in vivo*.

The present study has not yet addressed the functional roles of the other subunits of mammalian eIF3. We are currently trying to determine whether any of the non-core subunits (eIF3d, eIF3e, eIF3f, eIF3h, eIF3k, and eIF3l) have a role in enhancing the affinity of eIF3 for the 40 S ribosomal subunit. The baculovirus system of expressing eIF3 subunits is ideal for such studies. The purification of milligram quantities of eIF3 subcomplexes also enables us to assay *in vitro* the function of the subcomplexes in the various reactions in the initiation pathway such as stabilization of Met-tRNA$_i$ binding to 40 S ribosomal subunits and the synthesis of methionyl-puromycin. Many other initiation factors appear to interact with mammalian eIF3, such as eIF4G (13, 14), eIF4B (12), eIF1 (11), and eIF5 (15, 40). These associations also are amenable to characterization by the baculovirus system. The availability of purified eIF3 subcomplexes from baculovirus-infected Sf9 cells should help to define the minimum complex of eIF3 capable of forming a 40 S preinitiation complex that is able to scan to and identify the AUG initiation codon.

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REFERENCES

1. Benne, R., and Hershey, J. W. B. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3005–3009
2. Safer, B., Adams, S. L., Kemper, W. K., Berry, K. W., Lloyd, M., and Merrick, W. C. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 2584–2588
3. Schreier, M. H., Erni, B., and Staehelin, T. (1977) *J. Mol. Biol.* **116,** 727–753

[3] F. Peiretti, K. Block, and H.-P. Vornlocher, unpublished results.

4. Browning, K. S., Gallie, D. R., Hershey, J. W. B., Hinnebusch, A. G., Maitra, U., Merrick, W. C., and Norbury, C. (2001) *Trends Biochem. Sci.* **26,** 284
5. Hershey, J. W. B., and Merrick, W. C. (2000) in *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 33–88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Morris-Desbois, C., Rety, S., Ferro, M., Garin, J., and Jalinot, P. (2001) *J. Biol. Chem.* **276,** 45988–45995
7. Thompson, H. A., Sadnik, I., Scheinbuks, J., and Moldave, K. (1977) *Biochemistry* **16,** 2221–2230
8. Trachsel, H., and Staehelin, T. (1979) *Biochim. Biophys. Acta* **565,** 305–314
9. Goss, D. J., Rounds, D., Harrigan, T., Woodley, C. L., and Wahba, A. J. (1988) *Biochemistry* **27,** 1489–1494
10. Chaudhuri, J., Chowdhury, D., and Maitra U. (1999) *J. Biol. Chem.* **274,** 17975–17980
11. Fletcher, C. M., Pestova, T. V., Hellen, C. U. T., and Wagner, G. (1999) *EMBO J.* **18,** 2631–2639
12. Méthot, N., Song, M. S., and Sonenberg, N. (1996) *Mol. Cell. Biol.* **16,** 5328–5334
13. Lamphear, B. J., Kirchweger, R., Skern, T., and Rhoads, R. E. (1995) *J. Biol. Chem.* **270,** 21975–21983
14. Korneeva, N. L., Lamphear, B. J., Hennigan, F. L., and Rhoads, R. E. (2000) *J. Biol. Chem.* **275,** 41369–41376
15. Bandyopadhyay, A., and Maitra, U. (1999) *Nucleic Acids Res.* **27,** 1331–1337
16. Naranda, T., MacMillan, S. E., and Hershey, J. W. B. (1994) *J. Biol. Chem.* **269,** 32286–32292
17. Danaie, P., Wittmer, B., Altmann, M., and Trachsel, H. (1995) *J. Biol. Chem.* **270,** 4288–4292
18. Phan, L., Zhang, X., Asano, K., Anderson, J., Vornlocher, H. P., Greenberg, J. R., Qin, J., and Hinnebusch, A. G. (1998) *Mol. Cell. Biol.* **18,** 4935–4946
19. Valasek, L., Phan, L., Schoenfeld, L. W., Valaskova, V., and Hinnebusch, A. G. (2001) *EMBO J.* **20,** 891–904
20. Valasek, L., Hasek, J., Nielsen, K. H., and Hinnebusch, A. G. (2001) *J. Biol. Chem.* **276,** 43351–43360
21. Asano, K, Kinzy, T. G., Merrick, W. C., and Hershey, J. W. B. (1997) *J. Biol. Chem.* **272,** 1101–1109
22. Block, K. L., Vornlocher, H. P., and Hershey, J. W. B. (1998) *J. Biol. Chem.* **273,** 31901–31908
23. Brown-Luedi, M. L., Meyer, L. J., Milburn, S. C., Yau, M.-P. P., Corbett, S., and Hershey, J. W. B. (1982) *Biochemistry* **21,** 4202–4206
24. Falvey, A. K., and Staehelin, T. (1970) *J. Mol. Biol.* **53,** 1–19
25. Pincheira, R. Chen, Q. Huang, Z., and Zhang, J. T. (2001) *Eur. J. Cell Biol.* **80,** 410–418
26. Palecek, J., Hasek, J., and Ruis, H. (2001) *Biochem. Biophys. Res. Commun.* **282,** 1244–1250
27. Lin, L., Holbro, T. Alonso, G., Gerosa, D., and Burger, M. M. (2001) *J. Cell. Biochem.* **80,** 483–490
28. Méthot, N., Rom, E., Olsen, H., and Sonenberg, N. (1997) *J. Biol. Chem.* **272,** 1110–1116
29. Valasek, L., Nielsen, K. H., and Hinnebusch, A. G. (2002) *EMBO J.* **21,** 5886–5898
30. Asano, K., Phan, L., Anderson, J., and Hinnebusch, A. G. (1998) *J. Biol. Chem.* **273,** 18573–18585
31. Chaudhuri, J., Chakrabarti, A., and Maitra, U. (1997) *J. Biol. Chem.* **272,** 30975–30983
32. Bushell, M., Wood, W., Clemens, M. J., and Morley, S. J. (2000) *Eur. J. Biochem.* **267,** 1083–1091
33. Hinnebusch, A. G. (2000) in *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 185–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Phan, L., Schoenfeld, L. W., Valasek, L, Nielsen, K. H., and Hinnebusch, A. G. (2001) *EMBO J.* **20,** 2954–2965
35. Algire, M. A., Maag, D., Savio, P., Acker, M. G., Tarun, S. Z., Sachs, A. B., Asano, K., Nielsen, K. H., Olsen, D. S., Phan, L., Hinnebusch, A. G., and Lorsch, J. R. (2002) *RNA* **8,** 382–397
36. Valasek, L., Hasek, J., Trachsel, H., Imre, E. M., and Ruis, H. (1999) *J. Biol. Chem.* **274,** 27567–27572
37. Clemens, M. J., Bushell, M., and Morley, S. J. (1998) *Oncogene* **17,** 2921–2931
38. Marissen, W. E., and Lloyd, R. E. (1998) *Mol. Cell. Biol.* **18,** 7565–7574
39. Tee, A. R., and Proud, C. G. (2002) *Mol. Cell. Biol.* **22,** 1674–1683
40. Das, S., and Maitra, U. (2000) *Mol. Cell. Biol.* **20,** 3942–3950