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The Translation Initiation Factor eIF-4E Binds to a Common Motif Shared by the Translation Factor eIF-4γ and the Translational Repressors 4E-Binding Proteins

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Eukaryotic translation initiation factor 4E (eIF-4E), which possesses cap-binding activity, functions in the recruitment of mRNA to polysomes as part of a three-subunit complex, eIF-4F (cap-binding complex). eIF-4E is the least abundant of all translation initiation factors and a target of growth regulatory pathways. Recently, two human cDNAs encoding novel eIF-4E-binding proteins (4E-BPs) which function as repressors of cap-dependent translation have been cloned. Their interaction with eIF-4E is negatively regulated by phosphorylation in response to cell treatment with insulin or growth factors. The present study aimed to characterize the molecular interactions between eIF-4E and the other subunits of eIF-4F and to similarly characterize the molecular interactions between eIF-4E and the 4E-BPs. A 49-amino-acid region of eIF-4γ, located in the N-terminal side of the site of cleavage by *Picornaviridae* protease 2A, was found to be sufficient for interacting with eIF-4E. Analysis of deletion mutants in this region led to the identification of a 12-amino-acid sequence conserved between mammals and *Saccharomyces cerevisiae* that is critical for the interaction with eIF-4E. A similar motif is found in the amino acid sequence of the 4E-BPs, and point mutations in this motif abolish the interaction with eIF-4E. These results shed light on the mechanisms of eIF-4F assembly and on the translational regulation by insulin and growth factors.

A ubiquitous feature of all eukaryotic cellular mRNAs is the presence at their 5′ end of a cap structure, m7GpppX (where X is any nucleotide). The cap facilitates mRNA binding to ribosomes, a step which is rate limiting in translation (for reviews, see references 18 and 20). The cap is bound by the eukaryotic initiation factor 4F (eIF-4F) protein complex, which consists in mammals of three polypeptides: eIF-4E (a 25-kDa cap-binding protein), eIF-4γ (also known as p220 in mammals), and eIF-4A (a 46-kDa RNA helicase). eIF-4F, in conjunction with eIF-4B, exhibits RNA helicase activity. Evidence suggests that this activity mediates mRNA unwinding in the vicinity of the cap structure in an ATP-dependent manner and facilitates binding of the 40S ribosomal subunit (26, 33). The 40S subunit is then thought to scan the mRNA in the 5′-to-3′ direction until it encounters the initiator AUG codon. This series of steps constitutes the cap-dependent pathway of translation initiation (26, 33).

eIF-4E is the least abundant of all translation initiation factors, and its overexpression leads to deregulation of cell growth in HeLa cells (6) and to transformation of NIH 3T3 cells and CHO cells (5, 23) or rat primary fibroblasts, the latter in combination with v-*myc* or E1A (24). An attractive model for eIF-4E transforming activity is that overexpression of eIF-4E results in increased translation of mRNAs which are normally repressed, such as protooncogene mRNAs. eIF-4E activity is upregulated upon treatment of cells with growth factors, mitogens, and phorbol esters, which correlates with an increase in translation rates. Both eIF-4E and eIF-4γ are targets for growth-factor-induced phosphorylation (11, 27). Two proteins that interact with eIF-4E (4E-BP1 [also called PHAS-I] and 4E-BP2) and inhibit translation in a manner that is regulated by phosphorylation have been recently identified and characterized (25, 28). Phosphorylation of 4E-binding proteins (4E-BPs) by insulin and growth factors results in their dissociation from eIF-4E and relief of translational repression (25, 28).

Direct interaction between eIF-4E and the 4E-BPs was observed in vitro by the Far Western blot technique. Interactions between eIF-4E and other subunits of eIF-4F were evidenced by copurification over sucrose gradients or with affinity chromatography (8, 15, 35). In some species, such as *Saccharomyces cerevisiae* or wheat germ, eIF-4A is not present in the eIF-4F complex purified on a cap-affinity column, suggesting a loose association between eIF-4A and the other subunits of the complex (14, 22, 30). p220 is cleaved upon picornavirus infection (all genera except the cardioviruses and hepatoviruses), and this cleavage correlates with a drastic decrease in cap-dependent translation (9, 32). Virus translation occurs by a cap-dependent pathway through direct binding of the 40S ribosome subunit to the mRNA at an internal entry site (33).

Here, we report the identification by interaction cloning (3) of fragments of the human p220 protein that interact with eIF-4E and demonstrate a specific and direct interaction between the two translation factors by Far Western and coimmunoprecipitation assays. We have characterized both in mammals and *S. cerevisiae* a region of eIF-4γ involved in binding eIF-4E. This region contains a conserved motif, which is present also in the regulatory proteins 4E-BP1 and 4E-BP2 and is required for binding of these proteins to eIF-4E. These results suggest that eIF-4γ and 4E-BPs compete for binding to

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eIF-4E. The implications for the regulation of translation after hormone or growth factor stimulation are discussed.

MATERIALS AND METHODS

Plasmid constructions. For all construction steps involving the use of PCR, Vent DNA polymerase was used under standard conditions as recommended by the manufacturer (New England Biolabs). To create pAR-eIF-4E, the eIF-4E cDNA was excised from pet3b-4E (31) with *Nde*I and *Bam*HI. The cDNA fragment was blunt ended with the Klenow fragment of DNA polymerase I and cloned into pAR (delta RI)[59/60] (courtesy of Michael Blanar; see reference 3 for a description), digested with *Eco*RI, and blunt-ended with Klenow fragment. The orientation of the insert and that of the reading frame were confirmed by sequencing. The protein expressed by the recombinant vector is a fusion protein between the eIF-4E amino acid sequence and, at its N terminus, the sequence MDYKDDDDKARRASVEF. This sequence comprises a Flag peptide (DYK DDDDK), a site of cleavage by the enterokinase (between K and A), a recognition site (RRASV) for the heart muscle kinase (HMK), and the two amino acids (aa) encoded by the *Eco*RI site (EF).

The pGEX-p220 series of plasmids were constructed by cloning *Eco*RI DNA fragments prepared by PCR amplification of p220 sequences from the lambda phages into pGEX-2T[128/129] (a kind gift from Michael Blanar) that had been restricted by *Eco*RI. This vector was prepared by insertion between the *Bam*HI and *Eco*RI sites of pGEX-2T of an oligonucleotide encoding the peptide DYK DDDDKARRASV (see above).

KS-yeIF-4E was constructed by excising a 0.8-kb *Bam*HI-*Hin*dIII fragment containing the yeIF-4E coding sequences from pVTRP102-4E (2) and cloning it into pBluescript-KS (Stratagene) opened by *Bam*HI-*Hin*dIII. The eIF-4E coding sequence is under the transcriptional control of the T7 promoter.

KS-150 was constructed by excising a 4.2-kb *Hin*dIII cDNA fragment of TIF-4631 from pCG206 (13) and cloning it in the *Hin*dIII site of pBluescript-KS (Stratagene) in both orientations. KS-150Δ[441-470] was prepared by restriction of plasmid KS-150 (in the T3 orientation) with *Eco*RV followed by religation, which results in the deletion of the 3′ portion of the 150 gene. The resulting plasmid was further restricted at the *Eco*RI and *Spe*I sites of the polylinker and then subjected to blunt-end ligation with the *Bsa*HI-*Spe*I fragment of pCG206 (containing the 3′ portion of TIF-4631). Thus, the 150 cDNA sequences between the *Eco*RV and *Bsa*HI sites (87 bp) were replaced by 9 bp from the KS polylinker (*Eco*RV to *Eco*RI sites), resulting in the deletion of aa 441 to 470.

yeIF-4E and p150 genes were modified by epitope tagging as follows. An oligonucleotide containing, from 5′ to 3′, a *Hin*dIII site, a stop codon, the nucleotide sequence of the influenza virus hemagglutinin (HA) epitope YPYDVPDYASL (10, 21), and the last 17 nucleotides of yeIF-4E was used in conjunction with the M13 universal primer to amplify by PCR the yeIF-4E coding region, the template being KS-yeIF-4E. The resulting DNA fragment was digested with *Bam*HI and *Hin*dIII and cloned into pBluescript-KS opened by *Bam*HI and *Hin*dIII. A modified version of pBluescript-KS containing three consecutive copies of the HA epitope TAG sequence downstream of an initiation codon was prepared by cloning a *Hin*dIII-*Xba*I fragment excised from plasmid pACTAG-2 (a kind gift from Alain Charest, McGill University) and filled in by Klenow fragment into the *Sma*I site of KS (in the T7 promoter-driven orientation), generating KS-3HA-TAG. A 3.27-kb *Eco*RI cDNA fragment of TIF-4631 was excised from pCG 8.4 (13) and inserted in KS-3HA-TAG opened by *Eco*RI, generating KS-TAG-150. KS-TAG-150Δ[441-470] was prepared in two steps. First, the 650-bp *Hin*dIII-*Nco*I fragment of KS-150Δ[441-470] containing the 5′ end of the cDNA was exchanged with the 300-bp *Eco*RI-*Nco*I fragment from pCG8.4 in order to avoid the upstream stop codons present in the 5′ untranslated region of TIF-4631. The resulting plasmid was cleaved with *Cla*I and *Sac*I, and the insert was cloned into the *Eco*RV site of KS-3HA-TAG, resulting in KS-TAG-150Δ[441-470]. The tagged N-terminal deletion mutants of p150 were prepared by exonuclease III and mung bean nuclease nested deletions of KS-TAG-150 opened at *Pst*I (downstream of the triple TAG) and *Nco*I (at the 5′ end of p150).

Expression in *Escherichia coli* and protein purification. The glutathione *S*-transferase (GST)–p220 fusion proteins were prepared by transforming the corresponding pGEX-p220 vectors into BL21 cells and inducing the expression in exponentially growing cells with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial pellets obtained by centrifugation at 8,000 rpm for 5 min (Sorvall SS-34 rotor; Du Pont Instruments) were resuspended in Laemmli buffer and boiled for 5 min at 100°C. HMK-4E was expressed in *E. coli* K38 by the method of Tabor and Richardson (34). The recombinant HMK-4E was purified as described previously (7). Yields ranged from 5 to 10 mg of protein per liter of culture.

Screening of the lambda gt11 expression library. The lambda gt11 human placenta expression library was a generous gift from Morag Park. Screening of the expression library was performed as previously described (28). A total of 10^6 plaques were screened by using 2.5×10^5 cpm of ^{32}P-labeled HMK-4E per ml. Among the nine positive clones obtained, three (two of which were identical) corresponded to p220 fragments.

Western and Far Western blots. Protein extracts were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels (percentages indicated in figure legends) and transferred to nitrocellulose membranes. Western analyses were performed with rabbit polyclonal antibodies against GST or p220 (a gift from L. Carrasco) or a mouse monoclonal antibody against the HA-TAG (12CA5; a gift from M. Tremblay, McGill University). The eIF-4E probe was prepared for Far Western analysis by labeling 1 μg of purified eIF-4E with the catalytic subunit of bovine HMK (Sigma) as described previously (3). Processing of the nitrocellulose filters through a denaturation-renaturation cycle and hybridization (~12 h, 4°C) with the probe (10^5 cpm/ml) was as previously described (3). The intensities of the bands obtained on the Western and Far Western blots were quantitated by phosphorimager analysis. However, accurate quantitation was not possible because of the presence of degradation products of the fusion proteins.

Coimmunoprecipitation of in vitro-translated proteins. In vitro transcription and translation reactions were performed with T7 or T3 RNA polymerase and nuclease-treated rabbit reticulocytes as specified by the manufacturer (Promega). Translation reaction mixtures included [^{35}S]methionine for all proteins except for yeIF-4E, for which [^{3}H]leucine was used instead because of the low frequency of Met residues. Coimmunoprecipitation experiments were performed essentially as described previously (19). Briefly, labeled translation products were mixed and incubated at 30°C for 30 min. The reaction mixtures were diluted with 300 μl of ice-cold HND buffer {20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]–KOH [pH 8.0], 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 100 mM KCl, 10% glycerol, 0.4% Nonidet P-40, 1 mM dithiothreitol, 0.5% phenylmethylsulfonyl fluoride} and incubated for 2 h at 4°C with 1 μl of monoclonal antibody 12CA5 directed against the influenza virus HA epitope (HA-TAG). Protein G-Sepharose (Pharmacia) was subsequently added (15 μl per reaction mixture), and incubation was continued for 1 h. Immunoprecipitates were washed four times with HND buffer, boiled in Laemmli buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were fixed, enhanced by fluorography (En^{3}Hance; Du Pont Biotechnology Systems), and autoradiographed.

Poliovirus infection of HeLa cells and extract preparation. HeLa S3 cells grown in suspension were washed twice in phosphate-buffered saline at room temperature prior to infection with the Mahoney strain of poliovirus (multiplicity of infection = 30). Adsorption was achieved in Joklik medium supplemented with 1% heat-inactivated fetal calf serum, at room temperature for 45 min. After dilution with Joklik medium containing 5% fetal calf serum, incubation was continued for 4 h. Extracts were prepared essentially as described by Villa-Komaroff et al. (37). Cells were spun down in precooled glass jars, washed twice in isotonic buffer, and resuspended in hypotonic buffer (1.5 ml/ml of packed cells). The cells were allowed to swell for 5 min and then were lysed in a type B Dounce homogenizer. The extracts were spun down (5 min at 2,200 rpm, followed by 20 min at 10,000 rpm in an SS-34 rotor, with a Sorvall centrifuge [Du Pont Instruments]), and the supernatant was collected. After dialysis against buffer A {20 mM HEPES-KOH (pH 7.5), 100 mM KCl, 1.5 mM magnesium acetate [$Mg(OAc)_2$], 6 mM β-mercaptoethanol} and centrifugation at 10,000 × *g* for 10 min, the supernatant was aliquoted and frozen in dry ice-acetone.

RESULTS

eIF-4E interacts with 4E-BP1 and p220 in vitro. A radioactively labeled eIF-4E probe containing the phosphorylation site for HMK fused to eIF-4E was prepared by expression in *E. coli*, followed by purification over a cap column and in vitro phosphorylation. The HMK-4E probe allows for the detection of proteins interacting with eIF-4E after resolution by SDS-PAGE and transfer to nitrocellulose. To investigate the eIF-4E–p220 interaction, we prepared S10 extracts from either uninfected or poliovirus-infected HeLa cells. p220 is cleaved upon infection by poliovirus (see reference 32 for a review). This cleavage generates a set of three polypeptides migrating at ~130 kDa, which are recognized by antipeptide antibodies directed against the N terminus of p220, and one polypeptide of 110 kDa that is recognized by an antibody directed against the C terminus (38). As expected, the polyclonal anti-p220 antibody (a gift from L. Carrasco) recognized a cluster of proteins migrating at ~220 kDa in the uninfected extract and a cluster of proteins migrating at ~130 kDa and a protein of ~110 kDa in the infected extract (Fig. 1A). The proteins recognized by the HMK-4E probe had migration patterns similar to the ones detected by the antibody, but no 110-kDa polypeptide could be detected in the infected extract (Fig. 1B). Quantitation of the bands on the Western and Far Western blots demonstrated that the cleavage of p220 is accompanied not by a reduction in the affinity of the interaction with eIF-4E but rather by a two- to threefold enhancement, suggesting that all

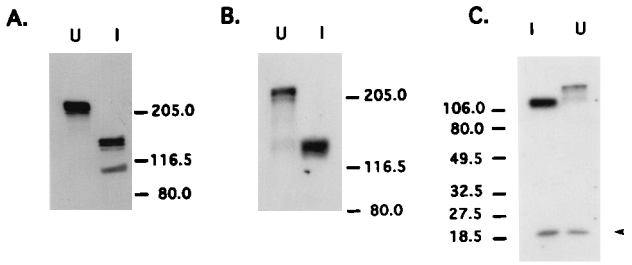

FIG. 1. Specificity of the interaction between eIF-4E and p220 detected by Far Western blot and effect of poliovirus infection. S10 extracts were prepared from HeLa S3 cells that were mock infected (U) or infected (I) with poliovirus for 4 h as described in Materials and Methods. (A) Western blot performed with a rabbit polyclonal antibody directed against p220 (gift from L. Carrasco). (B) Far Western blot performed with the same extracts and labeled meIF-4E as a probe. Both blots were prepared in parallel after electrophoresis on an SDS–6% polyacrylamide gel. (C) Far Western blot prepared in an identical manner as for panel B, except that electrophoresis was performed on an SDS–12% polyacrylamide gel. Positions of molecular mass standards (in kilodaltons) are indicated next to the blots.

the contacts established with eIF-4E are located within the N-terminal side of the cleavage site.

The only other major band detected by the HMK-4E probe in the S10 extracts after electrophoresis on an SDS–12% polyacrylamide gel corresponds to a ~20-kDa protein (Fig. 1C, arrowhead), which comigrates with 4E-BP1 (28), thereby demonstrating the specificity of this in vitro assay. (Note that on a 12% gel [Fig. 2C] the uncleaved p220 protein is more poorly transferred than from a 6% gel [Fig. 1B], explaining the lower intensity of the intact p220 band compared with the cleavage products.)

A 49-aa region of p220 interacts with eIF-4E. The HMK-4E protein was used as a probe to screen a λgt11 expression library from human placenta (28). Two of the cDNA clones isolated encoded 4E-BP1 and 4E-BP2 (28). Two other clones contained overlapping cDNA fragments of the p220 gene (encoding aa 240 to 773 and 355 to 874 [data not shown]). Two fragments of p220 cDNA sequences (encoding aa 240 to 773 and aa 355 to 773) were subcloned into pGEX-HMK (Fig. 2A). Although the fusion proteins were partially degraded, presumably because of the presence of protein-destabilizing PEST sequences

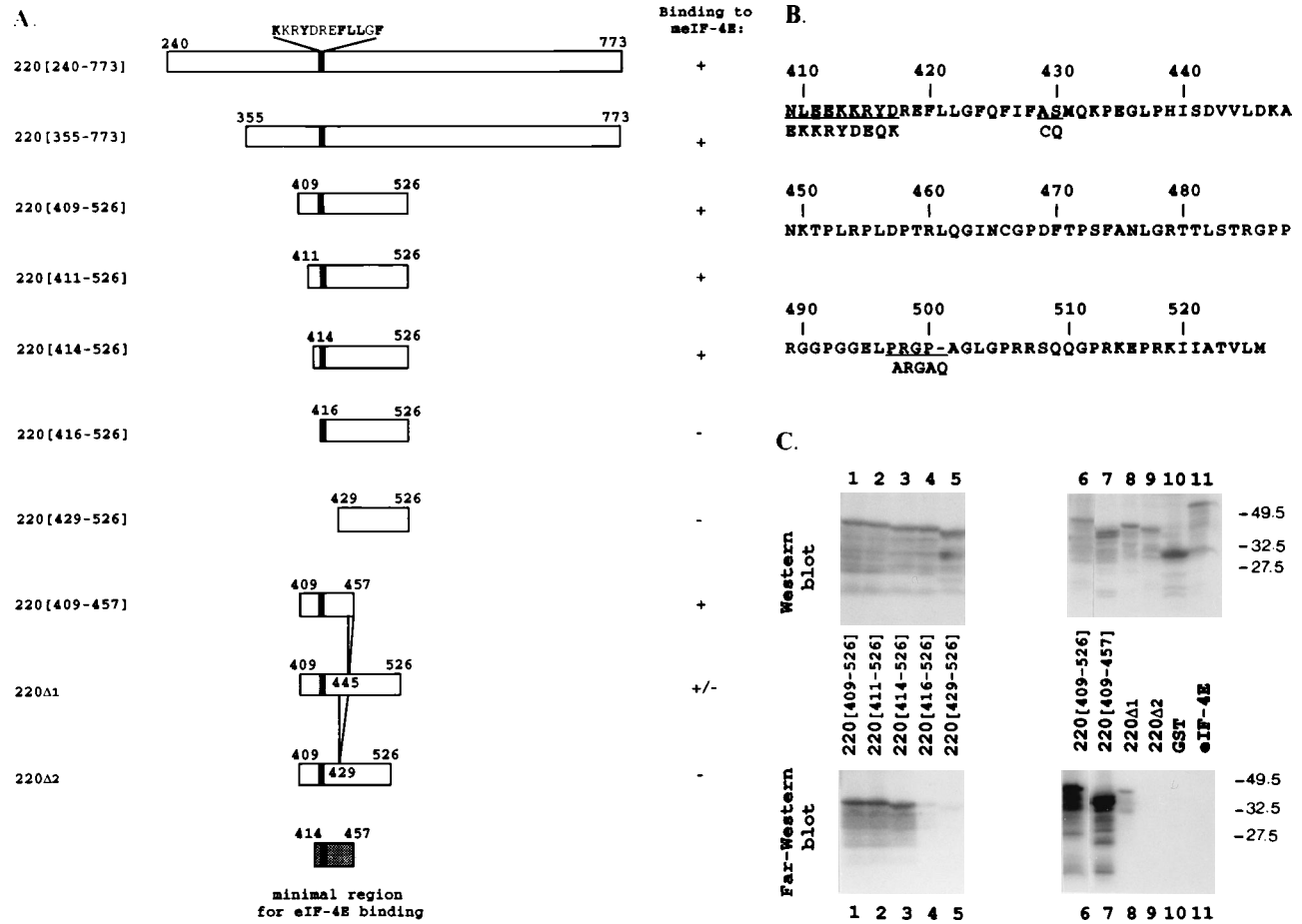

FIG. 2. Interaction between p220 deletion mutants and eIF-4E. (A) Deletion mutants of p220 expressed as fusion proteins with GST. The 12-aa stretch of homology (aa 413 to 424) between p220 and its yeast homolog p150 is indicated by a black bar. The capacity of each fusion protein to interact with mouse eIF-4E (meIF-4E) in a Far Western blot assay (see panel B) is indicated by + and −. (B) Amino acid sequence of p220[409-526]. Amino acid stretches differing from the published sequence (38) are underlined, and the published sequence is shown below. (C) The levels of expression of fusion proteins between GST and the p220 peptides are shown in the upper panel. A Western blot was performed with a rabbit polyclonal antibody against GST (prepared by A. Pause [27a]) on extracts prepared from IPTG-induced BL21 cells by direct lysis in Laemmli buffer. The interaction between the GST-p220 fusion proteins and meIF-4E was assayed by the Far Western technique (lower panel). The blots for the upper and lower panels were prepared in parallel with the same samples. For the Far Western blot assay, the nitrocellulose membrane was hybridized with the eIF-4E probe after denaturation and renaturation (Materials and Methods). Positions of molecular mass standards (in kilodaltons) are indicated next to the blots.

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wheat          HERVRYSRDQLLDLRKIT
                 ||  ||  ||  ||     |
human          EEKKRYDREFLLGFQFIF
               |||||||||||||||||||
rabbit         EEKKRYDREFLLGFQFIF
                 |   |    |||  |
yeast (4631)   HVKYTYGPTFLLQFKDKL
                 ||||||||||||||||||
yeast (4632)   SVKYTYGPTFLLQFKDKL
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FIG. 3. Conservation of a 12-aa motif in eIF-4γ throughout evolution. Comparison of the amino acid sequences of human and yeast proteins interacting with mammalian or yeast eIF-4E. The sequence of the wheat germ isozyme form of eIF-4γ is taken from reference 1. The sequence of p220 is from our sequenced fragment and corresponds to the amino acid sequence deduced from the published nucleotide sequence of human eIF-4γ (38). The sequence of the rabbit homolog was obtained from GenPept (L22090); the yeast homologs are the products of the TIF-4631 and -4632 genes (13).

in the p220 fragments (38), the interaction of the full-length product and of some degradation products with labeled eIF-4E could be clearly observed by Far Western analysis (data not shown).

We designed PCR primers to amplify shorter fragments of the p220 coding sequence (Fig. 2A), cloned them into the pGEX-HMK vector, and tested them for interaction with the eIF-4E probe. Expression of the different fusion proteins was assessed by Western analysis (Fig. 2C, upper panel). A 118-aa polypeptide, comprising aa 409 to 526 (220[409-526]), interacted with the probe (Fig. 2C, lane 1). The interaction between 220[409-526] and eIF-4E is specific since neither the GST protein, expressed from the parental vector at higher levels than the fusion protein, nor a GST–eIF-4E fusion protein was detected by the eIF-4E probe (Fig. 2C, lanes 10 and 11). The cDNA fragment corresponding to aa 409 to 526 was sequenced, and the deduced amino acid sequence is shown in Fig. 2B. There are several differences with the published sequence of p220 (underlined amino acids; the published sequence is shown below). One discrepancy (aa 409 to 417) is explained by the fact that the published amino acid sequence is out of frame with the published nucleotide sequence in this region. The other discrepancy could result from sequencing or cloning artifacts in this GC-rich region or from polymorphism.

To further delimit the eIF-4E interaction region, we deleted aa 458 to 526 from 220[409-526] with a *Bam*HI site present in the p220 sequence, generating the fusion protein 220[409-457]. This protein was recognized by HMK-4E as efficiently as 220[409-526] (Fig. 2C, lane 7). In the context of 220[409-526], deletions removing amino acids N terminal to aa 457 generated fusion proteins which interacted weakly (220Δ1, deletion of aa 445 to 457) or did not interact (220Δ2, deletion of aa 429 to 457) with HMK-4E compared with 220[409-526] (Fig. 2C, lanes 8 and 9; the expression levels of the three proteins were comparable [Fig. 2C, upper panel]). Taken together, these results suggest that the region of p220 encompassing aa 414 to 457 is sufficient for conferring eIF-4E-binding properties to the heterologous protein GST.

The 49-aa polypeptide (aa 409 to 457) contains in its N-terminal portion a 12-aa sequence (aa 413 to 424) of homology to p150, the yeast homolog of p220 (Fig. 3). This region contains five hydrophobic amino acids (Y-416, F-420, L-421 and L-422, F-424) which are conserved between p220 and p150 and will be referred to as the conserved motif. Deletion of this sequence (220[429-526]) generated a protein that was only very weakly recognized by HMK-4E (Fig. 2C, lane 5). Removal by progressive deletion of 5 of the 7 aa preceding the conserved motif in 220[409-526] (generating 220[411-526] and 220[414-526]) had no effect on the interaction with eIF-4E (Fig. 2C, lanes 2 and 3), but the fusion protein starting immediately at Tyr-416 had a much lower affinity for eIF-4E than 220[409-526] (Fig. 2C, compare lane 4 and lane 1). Taken together, these results suggest that the region of p220 encompassing aa 414 to 457, which contains a motif (aa 416 to 425) conserved in the yeast homolog p150, is necessary for binding of p220 to mouse eIF-4E.

Mapping of a minimal eIF-4E-binding region in yeast p150. To determine whether the domain of interaction of p220 with eIF-4E is conserved through evolution, we developed a coimmunoprecipitation assay to measure the interaction between in vitro-translated yeast eIF-4E and p150, the yeast p220 homolog (13). Sequences coding for the influenza virus HA epitope, YPYDVPDYASL (HA-TAG), were fused to the coding sequence of yeast eIF-4E and p150. Following incubation of in vitro-translated tagged eIF-4E (4E-TAG) with untagged p150 or luciferase as a control protein (Fig. 4B, upper panel, lanes 1 and 6), 4E-TAG was immunoprecipitated with a monoclonal antibody directed against the HA-TAG. The proteins complexed to the antibody were analyzed by SDS-PAGE. p150, but not luciferase, coprecipitated with 4E-TAG (Fig. 4B, lower panel, compare lanes 1 and 6). In the converse experiment, in which p150 but not eIF-4E was tagged, untagged eIF-4E coprecipitated with tagged 150 while luciferase did not (Fig. 4C, lanes 1 and 2). In a control experiment, the anti-HA-TAG antibody failed to precipitate either untagged p150 (Fig. 4B, lane 10) or untagged eIF-4E (Fig. 4C, lane 10) in the absence of tagged protein.

To map the eIF-4E binding site on p150, C-terminal deletion mutants of p150 were prepared by in vitro transcription of DNA templates linearized by digestion with restriction enzymes at various positions of the coding sequence, followed by in vitro translation (Fig. 4A). Untagged derivatives lacking the C-terminal 297 (150[1-655]) or 448 (150[1-504]) aa were coimmunoprecipitated efficiently with tagged eIF-4E (Fig. 4B, lanes 2 and 3), whereas a further truncation of 34 aa (150[1-470]) generated a mutant that was not coimmunoprecipitated (Fig. 4B, lane 8). As expected, a shorter mutant, 150[1-440], was not immunoprecipitated either (Fig. 4B, lane 4). Similar results were obtained when tagged C-terminal deletion mutants of p150 were incubated with untagged eIF-4E, since deletion to aa 440, but not aa 504, abolished the interaction with eIF-4E (Fig. 4C, lanes 4 and 5).

N-terminal deletions of p150 were prepared by exonuclease III and mung bean nuclease digestions of the TAG-150 plasmid after restriction enzyme digestion at the junction between the TAG and the 150 coding sequences. Products from various time points of digestion were religated and sequenced to determine the endpoint of the deletion (Fig. 4A). The tagged in vitro-translated products were tested for their capacity to form complexes with yeIF-4E in the coimmunoprecipitation assay (Fig. 4C). Deletion of the N-terminal 355 or 377 aa did not affect the interaction with eIF-4E (Fig. 4C, lanes 6 and 7). However, a mutant lacking the N-terminal 420 aa failed to coimmunoprecipitate with eIF-4E (Fig. 4C, lane 8). A further deletion of 3 aa (Tag150[424-952]) also generated a protein that did not interact with eIF-4E (Fig. 4C, lane 9). The results from the C-terminal and N-terminal deletion mutants define a region comprising aa 378 to 504 of p150 as the minimal sequence necessary for binding to eIF-4E in this assay. Centered in this region is the motif that is conserved between mammals and *S. cerevisiae* (aa 447 to 458 [Fig. 4A; see also Fig. 3]). Deletion of aa 441 to 470 (Fig. 4A) in the full-length p150 molecule resulted in the loss of the eIF-4E-binding activity

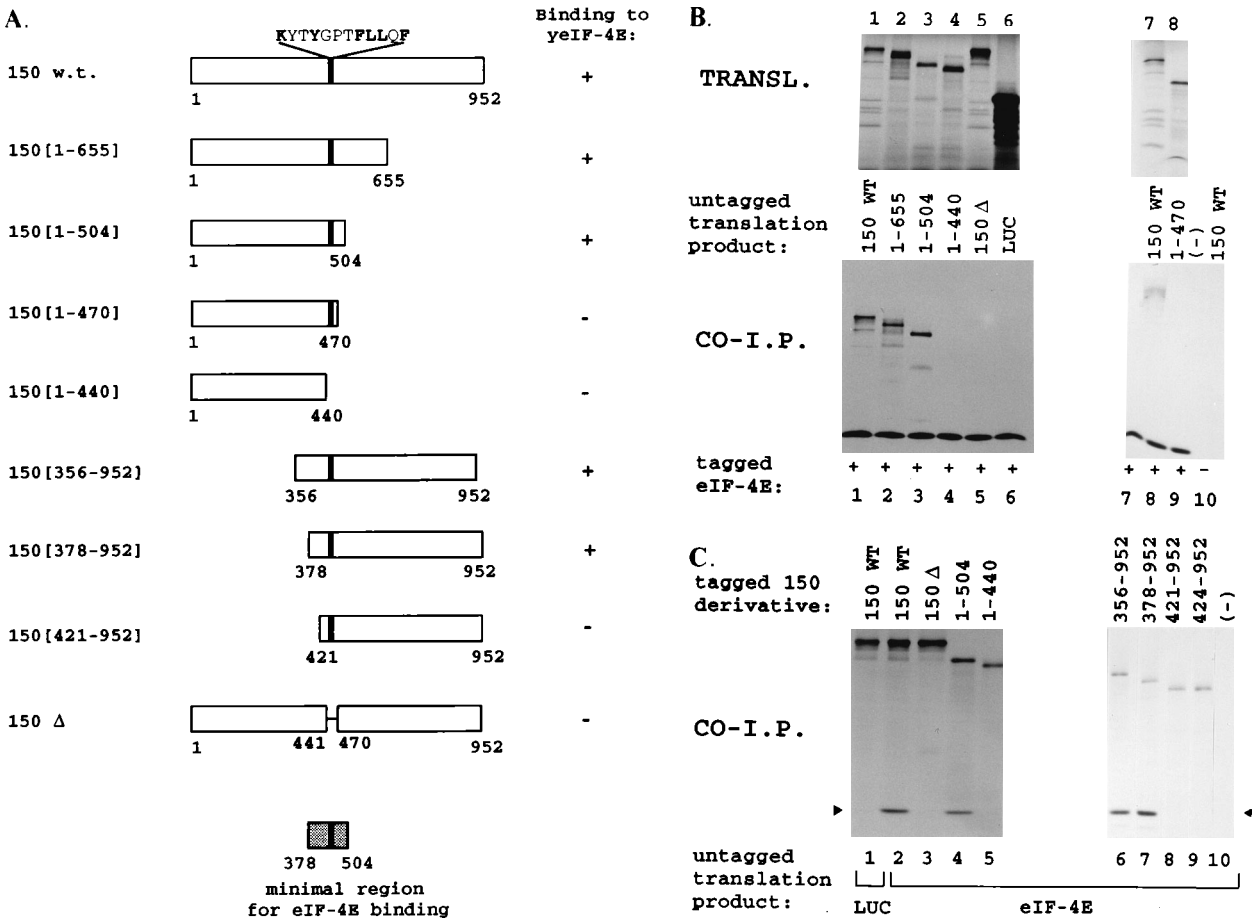

FIG. 4. Interaction between p150 deletion mutants and eIF-4E. (A) Deletion mutants of the yeast p150 coding sequence. The 12-aa region of homology with p220 (aa 447 to 458 of the product of the TIF-4631 gene) is shown as a black bar. The capacity of the mutants to interact with yeIF-4E in a coimmunoprecipitation assay (see panels B and C) is indicated by + and −. w.t., wild type. (B) Coimmunoprecipitation assay using HA-tagged yeIF-4E. [^{35}S]Met-labeled in vitro translation products of the untagged p150 mutants and of the negative control luciferase (LUC) were run on an SDS–12% polyacrylamide gel and detected by autoradiography after enhancement by fluorography (upper panel). Labeled translation products of the p150 derivatives were mixed with equivalent amounts of [^{3}H]Leu-labeled yeast eIF-4E and then immunoprecipitated with an antibody directed against the HA-TAG sequence (12CA5, a gift from M. Tremblay); this was followed by electrophoresis on an SDS–12% polyacrylamide gel and autoradiography (lower panel). The legend describing the untagged translation products obtained by in vitro translation and used in the coimmunoprecipitation assay applies to both the upper and lower panels. WT, wild type. (C) Coimmunoprecipitation assay using tagged p150 mutants. This assay is similar to that described for panel B, except that yeast eIF-4E was not tagged and the p150 derivatives carried three copies of the HA-TAG sequence at the N terminus. All 150 derivatives are therefore precipitated by the antibody directed against the HA-TAG sequence (12CA5, a gift from M. Tremblay), whereas eIF-4E (arrowhead) is present only when coimmunoprecipitated with the 150 derivatives. LUC, luciferase; WT, wild type.

(Fig. 4B, lane 5, and Fig. 4C, lane 3). Taken together, these results suggest the importance of the conserved motif in binding to yeIF-4E.

Integrity of the conserved motif is required for high-affinity interaction with eIF-4E. Since partial or total deletion of the conserved motif prevents the interaction of p150 or p220 with eIF-4E, we performed site-directed mutagenesis to investigate the importance of individual amino acids in the motif. Conserved amino acids were replaced by alanine residues (Fig. 5B). Phe-420 (mt4) or Phe-424 (mt6) could be replaced by Ala without any effect on eIF-4E binding (Fig. 5A, compare lanes 3 and 5 with lane 2). On the other hand, replacement of Leu-421 and Leu-422 by alanines yielded a mutant (mt5) that retained less than 2% of binding efficiency compared with the control 220[409-526] (Fig. 5A, compare lane 4 with lane 2). Replacement of Tyr-416 by Ala (mt2) reduced the interaction with eIF-4E to less than 5% of that observed with 220[414-526] (compare lanes 7 and 9). Replacement by a phenylalanine residue (mt3) was less disruptive, retaining ca. 20% of eIF-4E-binding activity (lane 6). Changing the nonconserved residue Arg-415 to Ala (mt1) did not affect binding (lane 8). These results demonstrate that at least two of the five hydrophobic residues cannot be replaced by Ala residues without a severe reduction in the affinity of the interaction with eIF-4E.

The motif of homology found in the minimal 4E-binding region of human and yeast eIF-4γ is also crucial for 4E-BP function. 4E-BP1 and 4E-BP2 were cloned by interaction cloning on the basis of their interaction with eIF-4E (28). Their interaction with eIF-4E is regulated by phosphorylation and correlates with inhibition of translation. This led to the proposal that binding of 4E-BPs to eIF-4E inhibits the formation of the eIF-4F complex. Sequence comparisons between 4E-BPs and the 4E-binding region in p220 do not reveal statistically significant overall identities, suggesting that the 4E-BPs and p220 are not evolutionarily related. However, a sequence very similar to the conserved motif of eIF-4γ is also found in the 4E-BPs. A total of 5 of the 6 aa conserved in this region between human and yeast eIF-4γ are present in the same arrangement in 4E-BP2 (K-413, Y-416, F-420, L-421–L-422),

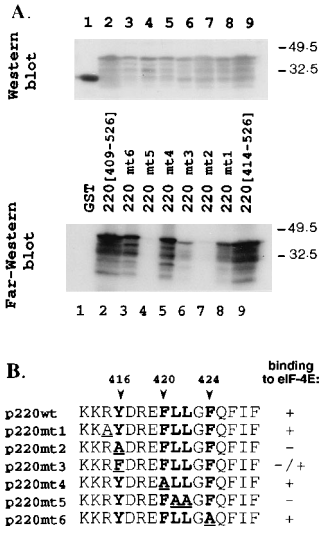

FIG. 5. Mutations in the conserved motif affect the interaction between eIF-4E and the high-molecular-weight subunit of eIF-4F. (A) Western blot (upper panel) and Far Western blot (lower panel) were performed with extracts prepared from BL21 cells induced with IPTG and expressing mutants of GST-p220 fusion proteins. Western blot analysis was performed with a rabbit polyclonal antibody against GST. Mutations 1 to 3 were introduced in the context of 220[413-526] by PCR amplification with a 5′ primer oligonucleotide carrying the mutation. Mutations 4 to 6 were introduced in the context of p220[409-526] by two cycles of PCR amplification with two complementary oligonucleotides in the region of the mutation. Positions of molecular mass standards (in kilodaltons) are indicated on the right. (B) Sequence of the p220 mutants used for panel A. The mutated amino acid is underlined; the conserved hydrophobic amino acids are in boldface.

and two additional charged amino acids are also present at similar positions in p220 and the 4E-BPs (Fig. 6A). To investigate the role of the conserved motif in 4E-BPs in binding to eIF-4E, we introduced mutations in this motif by site-directed mutagenesis. Deletion of most of the motif in 4E-BP1 or 4E-BP2 by removal of aa 54 to 62 generated proteins (GST-BP1Δ and GST-BP2Δ) that did not copurify with eIF-4E on a cap column as determined by Western analysis (Fig. 6B, lanes 3 and 6). We also introduced the double point mutation corresponding to 220mt5 (in which two conserved leucines are mutated to alanines) in both 4E-BP1 and 4E-BP2 (GST-BP1mt and GST-BP2mt [Fig. 6B, lanes 4 and 7]). By itself, this mutation is as effective in abrogating the interaction between the 4E-BPs and eIF-4E as deleting 9 aa from the motif. In conclusion, deletion or point mutations of amino acids that are shared by eIF-4γ and the 4E-BPs in the conserved motif are sufficient to abolish the interaction with eIF-4E.

DISCUSSION

We have characterized a 49-aa region in human eIF-4γ (p220, aa 409 to 457), which is necessary and sufficient for interaction with eIF-4E. In this region, the highest degree of conservation with the yeast homologs of p220, the products of the TIF-4631 and -4632 genes, corresponds to a 12-aa motif (6 aa of 12 conserved [Fig. 3]). Five of the six conserved amino acids are hydrophobic residues. A slightly higher degree of conservation (7 aa of 12) is observed between human eIF-4γ and the wheat germ counterpart, while the rabbit form is 100% identical to human eIF-4γ in this region (Fig. 5A). Significantly, this motif is also present in the eIF-4E-BPs 1 and 2 (4E-BP1 and -2) that have been recently identified as translational repressors (28).

Structure predictions for the 49-aa minimal region of interaction in p220 indicate possible alpha-helices corresponding to two stretches of amino acids (aa 409 to 430 and 439 to 449 according to the Garnier-Robson algorithm [12] and 411 to 433 and 443 to 449 according to the Chou-Fasman algorithm [4]). The first alpha-helical region encompasses the motif of homology which is rich in hydrophobic amino acids. The position and the extent of the potential alpha-helical stretches vary in the wheat germ and yeast forms and in the 4E-BPs. Sequence comparison did not reveal homology with proteins containing the coiled-coil, helix-loop-helix, or leucine-rich types of protein-protein interaction motifs.

Deletion mutagenesis of the motif of homology suggests that it is essential for the interaction between 4E-BPs and eIF-4E and for the interaction between eIF-4γ and eIF-4E. Point mutations affecting hydrophobic amino acids (Tyr-416 and the leucine doublet at positions 421 and 422 of human p220) abolished the interaction with eIF-4E in several assays (Far Western blot and copurification over a cap affinity column). Note that although replacement of the other hydrophobic residues by alanine residues did not detectably affect the interaction with eIF-4E, it is possible that replacement by amino acids other than alanines may affect binding. In addition, since the minimal fragment of p220 capable of strongly interacting with eIF-4E is 49 aa long (whereas the conserved motif spans only 12 aa), we cannot exclude that amino acids surrounding the motif participate in the interaction. Indeed, insulin-stimulated phosphorylation of 4E-BP1 is known to reduce its affinity for eIF-4E (28). The main insulin-induced phosphorylation site in adipocytes is Ser-64 (17), which is located 3 aa downstream of the conserved motif (Fig. 6A).

The results described here shed light on the mechanisms of regulation of cap-dependent translation initiation by growth factors, mitogens, and polypeptide hormones such as insulin.

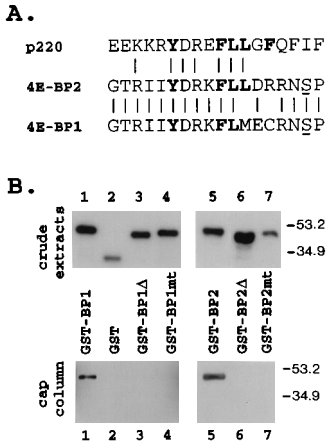

FIG. 6. Deletion or point mutation of the conserved motif in 4E-BPs abolishes the interaction with eIF-4E. (A) Comparison of sequences from p220 and 4E-BP1 and 4E-BP2 in the region of the conserved motif. The amino acid sequences shown correspond to residues 411 to 428 of human p220 (38) and to residues 49 to 66 of 4E-BP1 and 4E-BP2 (28). The amino acids in boldface correspond to the conserved position in p220, and identical residues or conservative changes in the 4E-BPs are indicated. The underlined Ser residue (Ser-64) in 4E-BP1 (PHAS-I) is phosphorylated upon insulin stimulation of adipocytes (17) and is conserved in 4E-BP2. (B) GST-fusion proteins of wild-type or mutated 4E-BP1 and 4E-BP2 were expressed in BL21 cells. The upper panel is a Western blot of crude bacterial extracts containing wild-type or mutant 4E-BPs. The lower panel is a Western blot with the same antibody after passage of crude extracts preincubated with eIF-4E over a cap affinity column. The antibody used in both panels is directed against GST. Positions of molecular mass standards (in kilodlatons) are indicated on the right.

We have previously postulated that the binding of 4E-BPs, which inhibit protein synthesis, to eIF-4E prevents the interaction of eIF-4E with p220 to form a biologically active eIF-4F complex. Consistent with this hypothesis, removal of the interaction motif in 4E-BP1 abolished the previously reported translational repression observed with purified 4E-BP1 in in vitro translation assays (16). Furthermore, the presence of a similar interaction motif both in the 4E-BPs and in p220 indicates that 4E-BPs repress translation by competing with eIF-4γ for binding eIF-4E. This possibility is supported by the capacity of 4E-BPs to compete with purified p220 for binding to eIF-4E in coincubation experiments (16). In addition, all the point mutants of eIF-4E assayed for interaction with eIF-4γ and 4E-BP1 were affected to the same degree in their interaction with the two proteins (data not shown).

Our results also demonstrate an interaction between recombinant p220 and eIF-4E expressed in bacteria, as well as between eIF-4E and the 4E-BPs, indicating that posttranslational modifications specific to eukaryotes are not required for this interaction. eIF-4E is phosphorylated in vivo in response to stimulation by a number of mitogens or differentiation-inducing agents (see references 11 and 27 for reviews). Phosphorylation of p220 is stimulated by phorbol myristate acetate or insulin in 3T3-L1 cells and by epidermal growth factor in epithelial cells (reference 27 and references therein). We tested the binding of labeled eIF-4E to proteins in whole-cell extracts prepared from HeLa cells treated with okadaic acid in coupled Far Western-Western blot assays and observed no difference in the magnitude of the interaction between eIF-4E and p220 forms from treated and untreated extracts (our unpublished observations). Further experiments will be necessary to examine whether phosphorylation of eIF-4E, or the combined phosphorylation of eIF-4E and p220, plays a role in the interaction between the two factors. Phosphorylation of these factors could also affect their interaction with other components of the translation initiation machinery.

Cleavage of p220 which occurs during poliovirus infection results in the separation of eIF-4E bound to the N-terminal cleavage product from the C terminus of p220 presumably bound to other translation factors. Among the translation factors that could interact directly with p220 are eIF-3, which was found to be associated with p220 (9, 36), and eIF-4A, the third subunit of eIF-4F. At present, it is not known which subunit of eIF-4F interacts with eIF-4A. We failed to detect an interaction between eIF-4E and eIF-4A in the Far Western assay (unpublished results), suggesting that eIF-4A might interact with p220. In this case, poliovirus infection would uncouple eIF-4E from eIF-4A, resulting in the inhibition of cap-dependent protein synthesis. The C-terminal part of p220 may still interact with eIF-4A and with the 40S ribosomal subunit, possibly via eIF-3, and mediate the association of the 40S subunit with internal sites on the RNA, thereby allowing translation of viral mRNAs by internal initiation (29).

In conclusion, we have characterized a motif crucial for the interaction with eIF-4E that is shared by several evolutionarily unrelated proteins. The point mutants of eIF-4γ and 4E-BPs which interfere with eIF-4E binding should provide useful tools for studies of the role of eIF-4E and 4E-BPs in translational initiation and in the regulation of cell growth, differentiation, and development.

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ADDENDUM IN PROOF

Lamphear et al. have recently shown, in agreement with our results, that eIF-4E interacts with the N-terminal portion of eIF-4γ. In addition, they showed that the carboxy-terminal half interacts with eIF-3 and eIF-4A (B. J. Lamphear, R. Kirchweger, T. Skern, and R. E. Rhoads, J. Biol. Chem., in press).

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