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4E-BP3, a New Member of the Eukaryotic Initiation Factor 4E-binding Protein Family*

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Translation initiation in eukaryotes is mediated by the cap structure (m^7GpppN, where N is any nucleotide) present at the 5′ end of all cellular mRNAs, except organellar. The cap is recognized by eukaryotic initiation factor 4F (eIF4F), which consists of three polypeptides, including eIF4E, the cap-binding protein subunit. The interaction of the cap with eIF4E facilitates the binding of the ribosome to the mRNA. eIF4E activity is regulated in part by two translational repressors, 4E-BP1 and 4E-BP2, which bind to it and prevent its assembly into eIF4F. We report here the isolation of 4E-BP3, a new member of the 4E-BP family. 4E-BP3 is homologous to 4E-BP1 and 4E-BP2, exhibiting 57 and 59% identity, respectively. The homology is most striking in the middle region of the protein, which contains the eIF4E binding motif and residues that are phosphorylated in 4E-BP1. 4E-BP3 is a heat stable protein that binds to eIF4E *in vitro* as well as *in vivo*. Further, 4E-BP3 overexpression specifically reduces eIF4E-dependent translation. The overlapping function and expression of the different 4E-BP family members imply that there is redundancy in this translational control mechanism, underscoring its importance.

Modulation of gene expression in cells is partly achieved by regulating translation rates. Translation initiation is the major target of such regulation in eukaryotic cells (1). The rate-limiting step in translation initiation is the binding of the 43 S preinitiation complex to the mRNA. This process is facilitated by the presence of the mRNA 5′-cap structure (2). Ribosome binding is mediated by eIF4F[1] (3, 4), which consists of three subunits: eIF4E, eIF4A, and eIF4G. eIF4F, in association with eIF4B, is thought to unwind the mRNA secondary structure in the vicinity of the cap to allow the binding of the 43 S preinitiation complex (reviewed in Refs. 4 and 5).

eIF4E is present in rate-limiting amounts in most cells (6, 7) (but see Rau *et al.* (8) for an exception in rabbit reticulocytes) and plays a central role in translational control (4, 5). The key role of eIF4E regulation in protein synthesis is underscored by the presence of two related eIF4E-binding proteins, 4E-BP1 and 4E-BP2 (9, 10). These proteins specifically inhibit eIF4E-dependent translation initiation (9) by competing with eIF4G for binding to eIF4E (11). Consequently, 4E-BPs prevent eIF4E association with eIF4G to form the eIF4F complex, resulting in the subsequent inhibition of the 43 S preinitiation complex binding to the mRNA. The competition between 4E-BPs and eIF4G is explained by the presence of a common eIF4E binding site in 4E-BPs and eIF4G (12).

The binding of 4E-BP1 and 4E-BP2 to eIF4E is controlled by their phosphorylation state. The underphosphorylated forms of 4E-BPs interact with eIF4E, whereas the hyperphosphorylated forms do not (9, 10, 13). Upon cell stimulation with serum, growth factors, or hormones, 4E-BP1 becomes hyperphosphorylated and dissociates from eIF4E to relieve the translational inhibition (9, 10, 14). The regulation of eIF4E activity by 4E-BPs is of particular significance considering the transforming potential of eIF4E (15). Indeed, the overexpression of 4E-BP1 and 4E-BP2 in cells transformed by eIF4E or v-*src* resulted in a significant reversion of the transformed phenotype (16). Here we present the molecular cloning and the characterization of 4E-BP3, a new member of the 4E-BP family of translational inhibitors.

EXPERIMENTAL PROCEDURES

Cloning of 4E-BP3 cDNA—The cDNA clone no. 87895 (Human Genome Sciences Inc.) was obtained from a prostate carcinoma cell line (LNCaP) EST cDNA library as described previously (17). Sequence alignment of 4E-BPs was performed using CLUSTAL W multiple sequence alignment program (version 1.7) (18).

Northern Blotting Analysis—Human 4E-BP3 mRNA distribution was analyzed by Northern hybridization using commercial blots (multiple tissue Northern blot 1 and 2, CLONTECH), with nucleotides 1–597 of the cDNA clone used as a probe, according to the manufacturer's instructions.

Antibodies—Anti-4E-BP3 antibody no. 1791 was raised in a rabbit against a synthetic peptide (CVTTPPTAPLSKLEE) comprising amino acids 67–80 of 4E-BP3. The peptide (2 mg) was cross-linked to keyhole limpet hemocyanin using a commercial kit (Pierce) according to the manufacturer's instructions. Anti-4E-BP3 antibody no. 1862 was raised in a rabbit against a GST-4E-BP3 fusion. Anti-eIF4E antibody no. 5853 was described previously (19).

Plasmids—pcDNA3-4E-BP3 was generated by cloning the coding sequence of 4E-BP3 into pcDNA3 (Invitrogen) using a polymerase chain reaction. Mutants of 4E-BP3 (Y40A or L45A) were generated by polymerase chain reaction mutagenesis and subcloned into pcDNA3. For N-terminally HA-tagged constructs, the coding sequence of 4E-BP3, 4E-BP3-Y40A, or 4E-BP3-L45A was subcloned into pcDNA3-HA (20). pcDNA3-FLAG-eIF4E

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[1] The abbreviations used are: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; EST, expressed sequence tag; RLUC, *R. reniformis* luciferase; FLUC, firefly luciferase; IRES, internal ribosome entry site; POLIRES, poliovirus internal ribosome entry site; GST, glutathione *S*-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; bp, base pair(s).

was constructed by cloning the entire coding sequence of mouse eIF4E into pcDNA3-FLAG using a polymerase chain reaction. The bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC was generated by subcloning the coding sequence for firefly luciferase (FLUC) from pGEM-LUC (Promega Biotech Inc.), the coding sequence for *Renilla reniformis* luciferase (RLUC) from pRL-CMV (Promega) and the complete poliovirus type II Lansing 5′-untranslated region (UTR) (poliovirus internal ribosome entry site, POLIRES) (nucleotides 1–737) (21) into pcDNA3.

Western Blotting Analysis—Polypeptides were resolved on SDS-15% polyacrylamide gels and transferred onto a 0.22-μm nitrocellulose membrane. The membrane was blocked for 16 h at 4 °C with 5% milk in Tris-buffered saline containing 0.5% Tween 20 (TBST). The membrane was incubated for 2 h with primary antibody 1862 (1:1000 in TBST with 1% bovine serum albumin) or 1791 (1:750 in TBST with 1% bovine serum albumin). Incubation with secondary antibody was performed with peroxidase-coupled donkey anti-rabbit Ig (Amersham Corp.) (1:5000 in TBST). Detection was performed with ECL (Amersham Corp.). For adsorption experiments, antiserum (10 μl) was preincubated on ice for 20 min with GST-heart muscle kinase tag (20 μg) or GST-4E-BP3 (20 μg) for antiserum 1862 or with the cognate peptide (20 μg) for antiserum 1791.

In Vitro Translation and Cap-affinity Assay—Capped RNA was synthesized with T7 RNA polymerase in the presence of the cap analog m^7GpppG (22). Wheat germ extract (Promega) (25 μl) was programmed with mRNA in the presence of [^{35}S]cysteine or methionine (25 μCi) according to the manufacturer's instructions. Following translation, extracts programmed with FLAG-eIF4E or 4E-BP3 mRNA were mixed and incubated on ice for 1 h to allow for protein interaction. Protein complexes were recovered at 4 °C for 60 min with 20 μl of m^7GDP-agarose resin (23) previously washed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The resin was washed three times with 1 ml of buffer A, resuspended in Laemmli (24) sample buffer, and boiled, and proteins were resolved by SDS-PAGE. Gels were processed for fluorography with En^{3}Hance (DuPont).

Cell Culture, Transfections, and Extract Preparation—HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were infected for 1 h in serum-free medium with recombinant vaccinia virus vTF7-3 (25, 26). Cells were then transiently transfected with pcDNA3-RLUC-POLIRES-FLUC (0.75 μg) and either pcDNA3, pcDNA3-4E-BP3, pcDNA3-4E-BP3-Y40A, or pcDNA3-4E-BP3-L45A (2 μg) using Lipofectin (Life Technologies, Inc.) (3.5 μl) as described previously (9). Cell extracts were prepared in Passive lysis buffer (Promega) 17 h after infection and assayed for RLUC and FLUC activity in a luminometer (BIOORBIT) using a dual-luciferase reporter assay system (Promega).

HeLa cells (6-cm dish) were transiently transfected with pcDNA3, pcDNA3-4E-BP3 (6 μg), or with pcDNA3-FLAG-eIF4E (3 μg) and pcDNA3-HA-La, pcDNA3-HA-4E-BP3, pcDNA3-HA-4E-BP3-Y40A, or pcDNA3-HA-4E-BP3-L45A (3 μg) using LipofectAMINE (Life Technologies, Inc.) (10 μl) according to the manufacturer's instructions. Cell extracts were prepared after 24 h by scraping cells in cold buffer A and subjecting the suspension to three freeze-thaw cycles. Cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was determined by the Bio-Rad assay.

LNCaP cells were grown in RPMI 1640 containing 10% fetal bovine serum. Cell extracts were prepared in buffer A as described above. LNCaP cell extract (5 mg/ml) was boiled for 8 min and then incubated on ice for 10 min. The precipitated material was removed by centrifugation, and Laemmli sample buffer was added to the supernatant before boiling for 5 min.

Co-immunoprecipitation and m^7GDP-Agarose Precipitation—For co-immunoprecipitation experiments, HeLa cells (6-cm dish) were lysed 24 h after transfection in 500 μl of cold Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and debris was spun down. Extracts were immunoprecipitated with anti-HA antibody HA.11 (BAbCO) (1.25 μg) for 1 h at 4 °C. Protein G-Sepharose (10 μl) was added, and the mixture was incubated for 1 h at 4 °C. After washing three times with 1 ml of Nonidet P-40 buffer, immunoprecipitates were eluted in Laemmli sample buffer, and proteins were subjected to SDS-PAGE.

For m^7GDP-agarose precipitation, 1.5 mg of LNCaP cell extract, prepared in buffer A as described above, was incubated with 30 μl of m^7GDP-agarose resin for 90 min at 4 °C. The resin was washed three times with 1 ml of buffer A and resuspended in Laemmli sample buffer, and proteins were subjected to SDS-PAGE.

Metabolic Labeling and Immunoprecipitation—LNCaP cells or transfected (pcDNA3 or pcDNA3-4E-BP3) HeLa cells (10-cm dish) were incubated for 5 h at 37 °C in serum-free Eagle's minimal essential medium containing 0.5 mCi/ml [^{32}P]orthophosphate (NEN Life Science Products; 3000 mCi/mmol). The medium was removed, and the cells were rinsed twice with phosphate-buffered saline. Cells were lysed in lysis buffer (10% glycerol, 50 mM Tris (pH 7.5), 60 mM KCl, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1% Triton X-100, 2 mM dithiothreitol, 50 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 1 mM EGTA, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C. Cells were harvested by scraping, and cell debris was removed by centrifugation. The extract was precleared at 4 °C for 1 h by incubation with preimmune serum (7 μl) bound to protein A beads (15 μl). The supernatant was transferred to a fresh tube together with 1862 antiserum (10 μl) bound to protein A beads (25 μl). Incubation end-over-end was carried out for 4 h at 4 °C. Beads were spun down and washed three times with lysis buffer, three times with radioimmune precipitation buffer, and three times with LiCl buffer (500 mM LiCl, 50 mM Tris (pH 7.5)). Immunoprecipitated material was subjected to SDS-PAGE, 15% gel, and transferred to a nitrocellulose membrane, which was dried and autoradiographed.

RESULTS

Cloning of 4E-BP3 cDNA—A cDNA for 4E-BP3 (Fig. 1*A*) was isolated from a LNCaP cDNA library. The cDNA is 698 bp long and consists of a 5′-UTR of 72 bp, an open reading frame of 300 bp, and a 3′-UTR of 326 bp. We do not know if the 5′-UTR of the cDNA is full-length. It is most probably close to full-length, as the size of the mRNA observed in Fig. 3 (800 bp) is close to the size of the cDNA (698 bp), taking into consideration the presence of a poly(A) tail on the mRNA. The 3′-UTR of the cDNA contains a polyadenylation signal (AGUAAA, *underlined* in Fig. 1*A*) located 9 nucleotides upstream of the poly(A) tail. The sequence, although not identical to the consensus polyadenylation sequence (AAUAAA), has been demonstrated to function in some viral cDNAs (27) and has not been excluded as a putative polyadenylation signal in eukaryotic mRNAs (28). The open reading frame encodes a predicted 100-amino acid protein with a molecular weight of 10,873. This putative polypeptide is highly homologous to the previously cloned 4E-BP1 and 4E-BP2 proteins, sharing 57 and 59% identity, respectively (Fig. 1*B*) (9). The homology between the 4E-BPs is highest in the middle portion, which contains the eIF4E binding region (12) (Fig. 1*B*, *boxed residues*). It is also of interest that all of the phosphorylation sites reported for rat 4E-BP1 (29) are conserved in 4E-BP2 and 4E-BP3 (Fig. 1*B*, denoted by *asterisks*), with the exception of a conservative change, serine 83 (in 4E-BP1) to a threonine in 4E-BP3. All of the phosphorylation sites contain a (Ser/Thr)-Pro motif.

Data base searches in GenBank™ identified several mouse ESTs that are predicted to encode for a protein highly homologous to the human 4E-BP3. The longest EST (GenBank™ accession no. W18851) is 623 bp long and could encode a protein of 101 amino acids with 86% identity to the human 4E-BP3 (Fig. 1*C*). It is therefore most probably the mouse homolog of human 4E-BP3.

Tissue Distribution of 4E-BP3—The tissue distribution of 4E-BP3 mRNA was analyzed by Northern blotting (Fig. 2). Two transcripts were detected, a major transcript of approximately 800 bp and a minor transcript of approximately 8.6 kilobase pairs. The smaller transcript most probably corresponds to the cDNA we have cloned here. Although 4E-BP3 RNA is expressed in almost every tissue, the levels of expression vary dramatically among tissues. 4E-BP3 expression is highest in skeletal muscle, heart, kidney, and pancreas, whereas there is very little expression in brain and thymus. The origin of the 8.6-kilobase pair transcript is not clear at present; it could represent a product of alternative splicing or a stable nuclear RNA containing intron sequences. A similar pattern of expression with two different transcripts was also observed for 4E-BP2

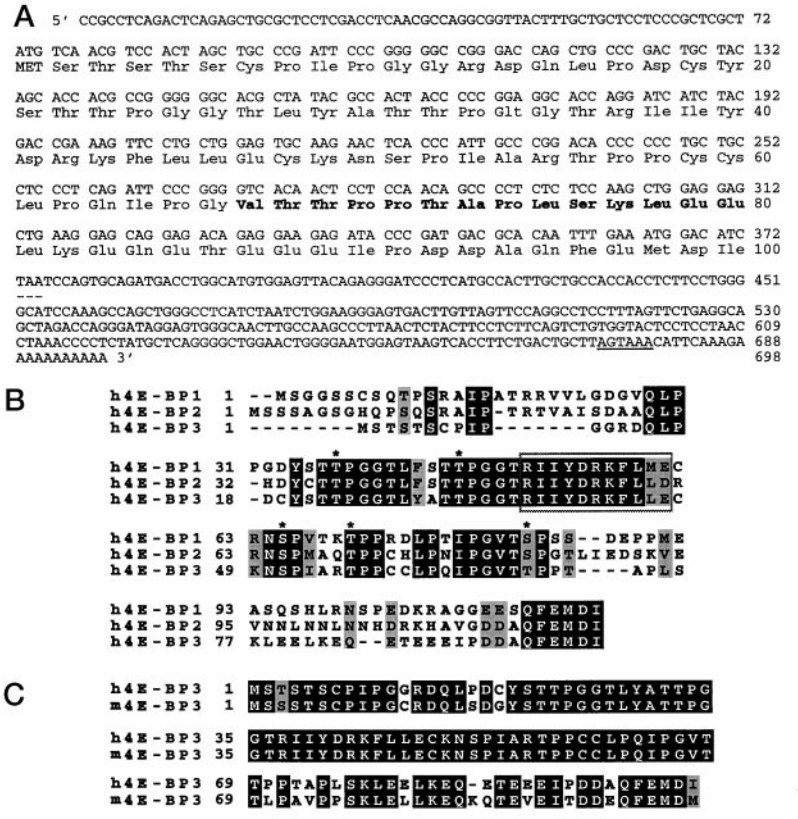

FIG. 1. **Sequence of 4E-BP3.** *A*, nucleotide and deduced amino acid sequence of human 4E-BP3. The polyadenylation signal is *underlined*. The sequence corresponding to the peptide used to generate antiserum 1791 is shown in *bold type*. *B*, sequence alignment of human 4E-BPs. Identical (*black box*) and conserved (*shaded box*) amino acids are highlighted. Amino acids involved in eIF4E binding are *boxed*. Phosphorylation sites of 4E-BP1 are denoted by *asterisks*. *C*, sequence alignment of human 4E-BP3 and mouse 4E-BP3 (EST W18851, GenBank™).

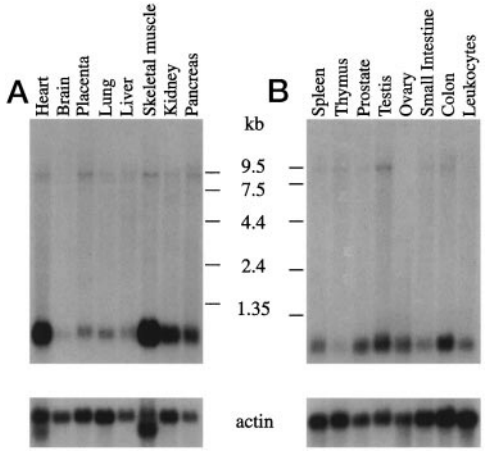

FIG. 2. **Northern analysis.** Tissue distribution of 4E-BP3 mRNA was analyzed using CLONTECH MTN1 (*A*) and MTN2 (*B*) Northern blots. The analysis was performed as described under "Experimental Procedures."

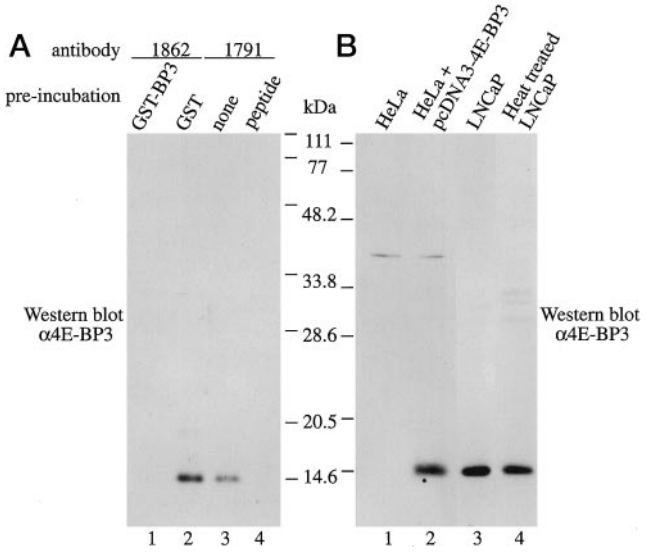

FIG. 3. **Identification of 4E-BP3 in cells.** *A*, an extract (100 μg) from LNCaP cells was resolved by SDS-PAGE, and Western blotting was performed with the following antisera: antiserum 1862 preadsorbed with GST-4E-BP3 (*lane 1*), antiserum 1862 preadsorbed with GST-HMK (*lane 2*), antiserum 1791 (*lane 3*), and antiserum 1791 preadsorbed with its cognate peptide (*lane 4*). *B*, an extract (20 μg) from HeLa cells transfected with pcDNA3 (*lane 1*) or with pcDNA3-4E-BP3 (*lane 2*) and total (*lane 3*) or heat-treated (*lane 4*) extract (250 μg) from LNCaP cells was resolved by SDS-PAGE. Western blotting was performed with antiserum 1791 (1:750).

(30), possibly pointing to an alternative splicing pattern conserved in 4E-BP3.

Identification of 4E-BP3 in Cells—To determine whether 4E-BP3 protein is expressed in cells, we used two different antisera raised in rabbits. The first (no. 1862) was raised against a GST-4E-BP3 fusion. The second (no. 1791) was raised against a peptide sequence derived from amino acids 67–80 in the C-terminal half of 4E-BP3 (Fig. 1*A*, *bold characters*), which is not conserved in the other 4E-BPs (Fig. 1*B*). Since 4E-BP3 cDNA was cloned from a cDNA library constructed from an LNCaP human prostate carcinoma cell line (31), an extract from this cell line was used to perform Western blotting. Each of the anti-4E-BP3 antisera detected a single polypeptide of 14.6 kDa (Fig. 3*A*, *lanes 2* and *3*). Moreover, the band corresponding to the 14.6-kDa polypeptide disappeared when the two antibodies were preincubated with their respective antigens (Fig. 3*A*, *lanes 1* and *4*). To further substantiate the authenticity of the 14.6-kDa polypeptide, we expressed 4E-BP3 cDNA in HeLa cells, which do not express 4E-BP3 at detectable levels (Fig. 3*B*, *lane 1*). A polypeptide which co-migrates with

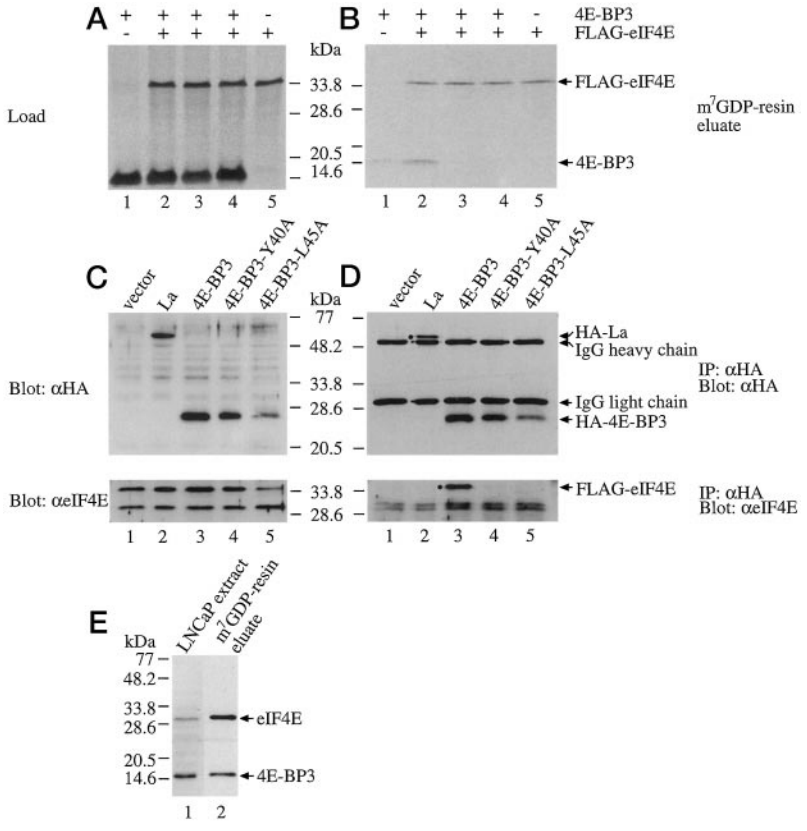

FIG. 4. **Interaction of 4E-BP3 with eIF4E.** *A*, *in vitro* synthesized capped mRNAs were translated in a wheat germ extract, and proteins were analyzed by SDS-PAGE as follows: *lane 1*, 4E-BP3; *lane 2*, 4E-BP3 + FLAG eIF4E; *lane 3*, 4E-BP3-Y40A + FLAG eIF4E; *lane 4*, 4E-BP3-L45A + FLAG-eIF4E; *lane 5*, FLAG-eIF4E. *B*, *in vitro* translated mixtures as described in *A* were incubated with m^7GDP-agarose resin. After washing, bound proteins were resolved by SDS-PAGE. *C*, HA-tagged protein expression plasmids, pcDNA3-HA (*lane 1*), pcDNA3-HA-La (*lane 2*), pcDNA3-HA-4E-BP3 (*lane 3*), pcDNA3-HA-4E-BP3-Y40A (*lane 4*), or pcDNA3-HA-4E-BP3-L45A (*lane 5*) were transfected into HeLa cells together with pcDNA3-FLAG-eIF4E. Total cell extracts (24 μg) were resolved by SDS-PAGE and analyzed by Western blotting for the presence of HA-tagged protein (*top panel*) or FLAG-eIF4E (*bottom panel*). *D*, HeLa cell extracts (300 μg) as described in *C* were immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS-PAGE. Western blotting analysis was performed using anti-HA antibody (*top panel*) and anti-eIF4E antibody (*bottom panel*). *E*, LNCaP cell extract was precipitated with m^7GDP-agarose, and the bound proteins were resolved by SDS-PAGE. Western blotting was performed using anti-4E-BP3 antibody 1791 (1:1000) and anti-eIF4E antibody 5853 (1:2500). *Lane 1*, LNCaP extract (150 μg); *lane 2*, m^7GDP-precipitated material.

4E-BP3 from LNCaP cells was observed in cells transfected with pcDNA3-4E-BP3 (Fig. 3*B*, compare *lane 2* to *lane 3*). Since 4E-BP1 and 4E-BP2 are heat stable proteins (9, 10), we wished to determine whether 4E-BP3 is also heat stable. An LNCaP cell extract was boiled, the soluble fraction subjected to SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. Probing with antisera 1791 demonstrated that 4E-BP3 is heat stable (Fig. 3*B*, *lane 4*).

Interaction with eIF4E—Since the eIF4E binding region in 4E-BPs is highly conserved in 4E-BP3 (Fig. 1*B*, *boxed residues*), it is most likely that 4E-BP3 would also interact with eIF4E. Mutants in the predicted eIF4E binding site were generated to further characterize this interaction. The design of the mutants was based on the results of Mader *et al.* (12) and from analysis of the conserved residues of the eIF4E binding motif found in all of the eIF4E-binding proteins reported so far (9, 32–34). In the first construct, Tyr-40 of 4E-BP3 was mutated to an alanine. An identical mutation in the eIF4E binding motif of eIF4G abolished its binding to eIF4E (12). The second construct was a mutant in which Leu-45 was changed to an alanine. Such a mutation was not analyzed by Mader *et al.*, in which only the two consecutive leucines of the motif were mutated to alanines, resulting in the loss of interaction with eIF4E (12).

To demonstrate an interaction between eIF4E and 4E-BP3, the proteins were synthesized by *in vitro* translation and analyzed for complex formation. Presence of the *in vitro* translated proteins was confirmed by SDS-PAGE, followed by fluorography (Fig. 4*A*). Very little wild type 4E-BP3 bound to the resin (Fig. 4*B*, *lane 1*). However, a significant amount of 4E-BP3 was retained on the resin in the presence of FLAG-eIF4E (4-fold increase as determined by PhosphorImager analysis, compare *lane 2* to *lane 1*). This demonstrates that, as with 4E-BP1 and 4E-BP2 (9), association of 4E-BP3 with eIF4E does not affect the binding of eIF4E to the cap structure. Analysis of the binding was also performed using 4E-BP3 containing mutations in the eIF4E binding region. The mutants showed no binding to FLAG-eIF4E (Fig. 4*B*, *lanes 3* and *4*). As expected, FLAG-eIF4E bound to the resin (Fig. 4*B*, *lane 5*).

To confirm the interaction of 4E-BP3 and eIF4E *in vivo*, co-immunoprecipitations were performed on extracts from HeLa cells transiently transfected with HA-tagged 4E-BP3. The light chain of the anti-HA antibody co-migrates with the endogenous eIF4E on SDS-PAGE, rendering detection of co-immunoprecipitated endogenous eIF4E difficult. To circumvent this problem, FLAG-tagged eIF4E, which migrates more slowly than does endogenous eIF4E, was co-expressed with HA-tagged 4E-BP3, 4E-BP3-Y40A, 4E-BP3-L45A, or La, an unrelated RNA-binding protein (35). Expression of the proteins was confirmed by Western blotting with αHA and αeIF4E (Fig. 4*C*). Immunoprecipitations were carried out using a monoclonal anti-HA antibody, and the immunoprecipitates were assayed by Western blotting for the presence of eIF4E and HA-tagged protein. FLAG-eIF4E was not co-immunoprecipitated when cells were co-transfected with the pcDNA3-HA vector (Fig. 4*D*, *lane 1*), nor in the presence of HA-La (Fig. 4*D*, *lane 2*). However, FLAG-eIF4E was co-immunoprecipitated in the presence of HA-4E-BP3 (Fig. 4*D*, *lane 3*). 4E-BP3 mutants Y40A and L45A failed to co-immunoprecipitate FLAG-eIF4E (Fig. 4*D*, *lanes 4* and *5*). The expression of the Y40A and L45A constructs was approximately 2–4-fold less efficient as compared with that of the wild type construct (Fig. 4*C*, compare *lanes 4* and *5* to *lane 3*), which is expected to affect the amount of FLAG-eIF4E brought down in the assay. However, this cannot explain the complete absence of FLAG-eIF4E.

Association of endogenous eIF4E with 4E-BP3 was also examined in LNCaP cells. The presence of eIF4E and 4E-BP3 in LNCaP cell extract was demonstrated by Western blotting with αeIF4E and α4E-BP3 (Fig. 4*E*, *lane 1*). eIF4E was precipitated from the extract using m^7GDP-agarose resin, and the presence of endogenous eIF4E and 4E-BP3 in the precipitate was ob-

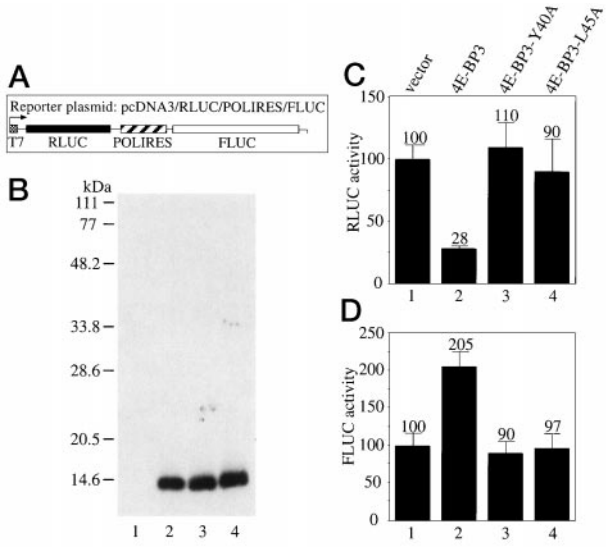

FIG. 5. **4E-BP3 inhibits cap-dependent translation in vivo.** HeLa cells were infected with recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase, and then transfected with pcDNA3-RLUC-POLIRES-FLUC and pcDNA3-4E-BP3. *A*, pcDNA3-RLUC-POLIRES-FLUC reporter vector (see "Experimental Procedures" for details). *B*, Western blot analysis of transfected cell extracts (10 μg) for the presence of 4E-BP3 with anti-4E-BP3 1791 (1:750). *Lane 1*, pcDNA3; *lane 2*, pcDNA3-4E-BP3; *lane 3*, pcDNA3-4E-BP3-Y40A; *lane 4*, pcDNA3-4E-BP3-L45A. *C* and *D*, RLUC and FLUC activity was measured after 17 h as described under "Experimental Procedures." The activity of the RLUC cistron is reported in *C*, and the activity of the FLUC cistron is reported in *D*. The RLUC and FLUC activity of pcDNA3 transfected cells is set at 100%. Each experiment was carried out twice in triplicate. The *error bars* represent the standard deviation of the mean.

served by Western blotting (Fig. 4*E*, *lane 2*).

Inhibition of Cap-dependent Translation in Vivo—To determine whether 4E-BP3 inhibits cap-dependent translation, a transient expression assay was used in which HeLa cells were infected with the recombinant vaccinia virus vTF7-3 (25, 26), and co-transfected with 4E-BP3 wild type or mutant constructs and a reporter plasmid. The reporter construct consisted of two cistrons separated by the POLIRES (Fig. 5*A*). Translation of the RLUC cistron is cap-dependent, whereas that of the FLUC cistron is directed by the poliovirus IRES and is therefore cap-independent (36). Expression of the 4E-BP3 proteins was assayed by Western blotting, and the results show a similar expression level for all the constructs (Fig. 5*B*). The level of expression of RLUC and FLUC was set at 100% when the reporter plasmid was co-transfected with an empty vector (pcDNA3) (Fig. 5, *C* and *D*, *column 1*). When co-transfected with a vector containing the coding sequence for 4E-BP3, the expression of RLUC was decreased by 72% (Fig. 5*C*, *column 2*), whereas the expression of FLUC showed a 2-fold increase (Fig. 5*D*, *column 2*). The Y40A mutant 4E-BP3 as well as the L45A mutant lost their capacity to inhibit cap-dependent translation (Fig. 5*C*, *column 3*, 110% of the control; *column 4*, 90% of the control, respectively). The two mutants did not affect cap-independent translation (Fig. 5*D*, *columns 3* and *4*), in contrast to wild type 4E-BP3.

Metabolic Labeling of 4E-BP3—To demonstrate that 4E-BP3 is a phosphoprotein, metabolic labeling of transfected HeLa cells or LNCaP cells was performed using [^{32}P]orthophosphate. Analysis of the immunoprecipitated material shows that 4E-BP3 was labeled with ^{32}P in HeLa cells transfected with pcDNA3-4E-BP3 (Fig. 6, *lane 2*), but not in HeLa cells transfected with pcDNA3 (Fig. 6, *lane 1*). Moreover, the endogenous 4E-BP3 was also found to be labeled in LNCaP cells (Fig. 6, *lane 3*). Taken together the data indicate that, as with 4E-BP1 and 4E-BP2 (9, 10, 13), 4E-BP3 is also a phosphoprotein.

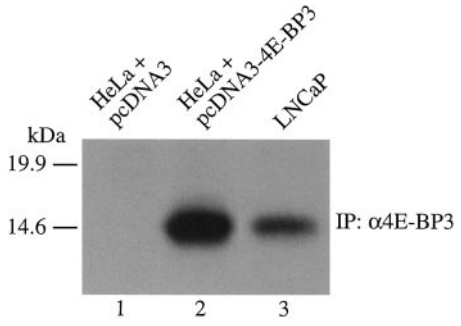

FIG. 6. **Metabolic labeling of 4E-BP3.** HeLa cells transfected with pcDNA3 (*lane 1*) or with pcDNA3-4E-BP3 (*lane 2*), or LNCaP cells (*lane 3*) were labeled with [^{32}P]orthophosphate, and 4E-BP3 was immunoprecipitated using 1862 antiserum as described under "Experimental Procedures."

DISCUSSION

We have isolated a new cDNA encoding for a protein that shares a high degree of identity with the translational inhibitors 4E-BP1 and 4E-BP2 (9) and was thus termed 4E-BP3. Data base searches (NCBI data base and Human Genome Sciences data base) have not revealed any homologies other than to 4E-BP1, 4E-BP2, and to ESTs encoding for the three 4E-BPs.

4E-BP3 was identified by Western blotting as a polypeptide having an apparent molecular mass of 14.6 kDa, which differs from the predicted molecular mass of 10.9 kDa. However, overexpression of 4E-BP3 in HeLa cells and *in vitro* translation of 4E-BP3 also yielded a polypeptide having an apparent molecular mass of 14.6 kDa, suggesting that the cDNA indeed encodes for a full-length protein. This type of aberrant migration is also observed for 4E-BP1 and 4E-BP2 (9, 37, 38) and is thought to be due to the high proline content of the proteins, which might affect their mobility in SDS-PAGE (39). Since the 14.6-kDa polypeptide is recognized by two different antibodies and shares the characteristic heat stability of 4E-BP1 and 4E-BP2 (9, 13, 38), it is reasonable to conclude that it is the third member of the 4E-BP family.

Interaction of 4E-BP3 with eIF4E was demonstrated by co-immunoprecipitation and by m^{7}GDP-affinity chromatography, both in cell extracts and with *in vitro* translated proteins. The interaction was also analyzed by generating mutants in the eIF4E binding motif of 4E-BP3. The two mutants (Y40A and L45A) did not show any detectable interaction with eIF4E in the assays performed. This result was expected for the Y40A mutant, as it was previously shown that such a mutation in eIF4G abolishes binding to eIF4E (12). The inability to detect an interaction of the L45A mutant with eIF4E demonstrates that the first of the two consecutive leucine residues in the eIF4E binding motif, alone dramatically affects the interaction. This result is not surprising as the first leucine is absolutely conserved in all of the eIF4E-binding proteins from yeast to human (9, 32–34, 40).

The effect of the Y40A and L45A mutants on translation was also assessed in a transient expression assay in which 4E-BP3 was shown to inhibit eIF4E-dependent translation by 72%. As expected from mutants with an impaired ability to bind to eIF4E, no effect was observed on eIF4E-dependent translation. The mutants also abolished the stimulation (2-fold) of IRES-mediated translation by 4E-BP3. Such a stimulation has not been observed in earlier reports with 4E-BP1 and 4E-BP2 (9, 41). The stimulation is specific as the mutants that are defective in binding to eIF4E, although expressed at a level similar

to that of wild type 4E-BP3 (Fig. 5*B*, compare *lanes 3* and *4* to *lane 2*), lost the ability to stimulate IRES-dependent translation. Since 4E-BPs and eIF4G compete for a limiting amount of eIF4E, it is possible that the high level of overexpression of 4E-BP3 in this system (Fig. 5*B*, *lane 2*) releases more of eIF4G that can participate in IRES-mediated translation and thus increase the expression of FLUC.

4E-BP3 mRNA tissue expression differs from that of 4E-BP1 and 4E-BP2 (30, 37). This may suggest that the regulation of eIF4E function by the 4E-BPs is modulated in a tissue-specific manner and is dependent upon the relative abundance of each 4E-BP. Since the 4E-BPs share biochemical and functional characteristics, it is likely that their relative activity in a given tissue or cell type is regulated post-translationally. It is well established that 4E-BP1 activity is regulated via phosphorylation (9, 14, 42, 43). Since 4E-BP3 is a phosphoprotein, it is highly likely that its activity is also regulated by its phosphorylation state. A differential regulation of the phosphorylation of the 4E-BPs could explain the redundancy of their function. However, recent reports show that 4E-BP1 and 4E-BP2 behave similarly in response to insulin and cAMP treatment (38) or to adenovirus infection (13, 44). Therefore, the overlapping expression of the 4E-BPs may also reflect a safeguard mechanism. In support of such a hypothesis, a mouse knock-out of 4E-BP1 failed to show any phenotype[2] (45), suggesting that the overlapping expression of other 4E-BPs may compensate for the disrupted 4E-BP1. This underscores the importance of 4E-BPs in regulating eIF4E-dependent translation initiation.

In conclusion, the data presented show that 4E-BP3 shares biochemical and functional characteristics with 4E-BP1 and 4E-BP2. To examine whether the 4E-BPs are differentially regulated, it will be important to determine if 4E-BP3 behaves in a manner similar to 4E-BP1 and 4E-BP2 in response to extracellular stimuli. Specifically, analysis of the phosphorylation of the 4E-BPs under various conditions will be required to ascertain if differences among the 4E-BPs exist.

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[2] K. Tsukiyama-Kohara and N. Sonenberg, unpublished data.

NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

4E-BP3, a New Member of the Eukaryotic Initiation Factor 4E-binding Protein Family

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