Age-specific CUGBP1-EIF2 Complex Increases Translation of CCAAT/Enhancer-binding Protein β in Old Liver*

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The RNA-binding protein CUGBP1 regulates translation of proteins in a variety of biological processes. In this study, we show that aging liver increases CUGBP1 translational activities by induction of a high molecular weight protein-protein complex of CUGBP1. The complex contains CUGBP1, subunits α, β, and γ of the initiation translation factor eIF2, and four proteins of the endoplasmic reticulum, eR90, CRT, eR60, and Grp78. The induction of the CUGBP1-eIF2 complex in old livers is associated with the elevation of protein levels of CUGBP1 and with the hyper-phosphorylation of CUGBP1 by a cyclin D3-cdk4 kinase, activity of which is increased with age. We have examined the role of the elevation of CUGBP1 and the role of cyclin D3-cdk4-mediated phosphorylation of CUGBP1 in the formation of the CUGBP1-eIF2 complex by using CUGBP1 transgenic mice and young animals expressing high levels of cyclin D3 after injection with cyclin D3 plasmid. These studies showed that both the increased levels of CUGBP1 and cdk4-mediated phosphorylation of CUGBP1 are involved in the age-associated induction of the CUGBP1-eIF2 complex. The CUGBP1-eIF2 complex is bound to C/EBPβ mRNA in the liver of old animals, and this binding correlates with the increased amounts of liver-enriched activator protein and liver-enriched inhibitory protein. Consistent with these observations, the purified CUGBP1-eIF2 complex binds to the 5′ region of C/EBPβ mRNA and significantly increases translation of the three isoforms of C/EBPβ in a cell-free translation system, in cultured cells, and in the liver. Thus, these studies demonstrated that age-mediated induction of the CUGBP1-eIF2 complex changes translation of C/EBPβ in old livers.

Recent publications revealed a significant role of RNA-binding proteins in the development of the aging phenotype in senescent cultured cells (1–3). Senescent fibroblasts change the activity of the RNA-binding protein HuR, leading to alterations in the stability of several mRNAs that translate cell cycle proteins and proteins involved in replicative senescence (3). We have shown that the induction of p21 in senescent fibroblasts is mediated by CUGBP1, which binds to the 5′ region of p21 mRNA and increases translation of p21 (4). Although the HuR-mediated stabilization of mRNA is regulated mainly by control of the cytoplasmic concentration of HuR (2, 3), the activities of CUGBP1 are regulated in regenerating livers and in senescent fibroblasts by phosphorylation (4–7). Despite some progress in studies of age-associated changes of RNA-binding proteins in cultured cells, the role of these proteins in the development of the aging phenotype in animals has not been examined.

It has been shown that aging liver increases translation of a dominant negative isoform of C/EBPβ (8). A single C/EBPβ mRNA produces three isoforms through alternative translation from three in-frame AUG codons as follows: full-length (FL), liver-enriched activator protein (LAP), and liver-enriched inhibitory protein (LIP). Whereas FL and LAP isoforms regulate promoters through interactions with chromatin remodeling complexes SWI/SNF and p300 (9), LIP lacks the region that interacts with these complexes and functions as a dominant negative molecule that inhibits the activities of full-length C/EBP proteins (10). Translation of LIP is increased in proliferating livers and activates expression of cyclins (6, 11). C/EBPβ mRNA contains an upstream open reading frame (uORF) that plays a crucial role in the regulation of translation of C/EBPβ isoforms. Experimental data from Johnson and co-workers (12) showed that the uORF inhibits total translation of C/EBPβ. However, other publications showed that the initiation of translation on the AUG codon of uORF is required for translation of LIP (13, 14). A third mechanism for the regulation of C/EBPβ translation was suggested by investigations of RNA-binding proteins. We and others have found that the interaction of CUGBP1 with the 5′ region of C/EBPβ mRNA activates translation of LIP (5–7).

In this study, we show that old livers accumulate a high molecular weight CUGBP1-eIF2 complex, which binds to the 5′ region of C/EBPβ mRNA and increases translation of LIP.
and LAP. The overall induction of LIP translation by the complex is much greater than the induction of LAP, leading to alterations in the LIP/LAP ratio. This complex consists of at least eight proteins, four that are involved in translation and four other proteins that are components of the endoplasmic reticulum. We found that aging liver increases the CUGBP1-eIF2 complex through cdk4-mediated phosphorylation of CUGBP1 and by elevation of protein levels of CUGBP1. Examination of the mechanisms by which the complex increases translation of C/EBPβ showed that the complex recruits a larger number of ribosomes to translate C/EBPβ mRNA.

**MATERIALS AND METHODS**

**Animals**

Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439). In this study, we have used young (4–6 months) and old (22–24 months) mice. Livers were harvested and kept at −80 °C. Expression of cyclin D3 in livers of young mice was increased by injection of pAdTrack-cyclin D3 plasmid and LAP-ORF sequences within the C/EBP uORF and LAP-ORF RNA oligomers. Locations of uORF and RNA Probes are shown below.

**RNA Probes**

Gel shift and UV cross-link assays were performed with uORF and LAP-ORF RNA oligomers. Locations of uORF and LAP-ORF sequences within the C/EBPβ mRNA and nucleotide sequences of these probes are shown in Fig. 2.

**UV Cross-link Assay**

UV cross-linking analysis was described in our previous paper (4, 6, 15). RNA probes were labeled by [γ-32P]ATP and T4 kinase. Equal amounts of radioactive RNA probes were incubated with proteins for 30 min at room temperature and subjected to UV treatment for 5 min at 125 mJ. Reaction mixtures were loaded on denaturing 8–16% gradient PAGE (Bio-Rad). Proteins were transferred onto the membrane and autoradiographed. The membranes were stained with Coomassie Blue to verify protein loading.

**UV Cross-link Immunoprecipitation Assay**

Proteins were linked to the ORF-LAP probe by UV and then precipitated using antibodies to CUGBP1. After washing, immunoprecipitates were loaded on a denaturing gel, transferred on membrane, and autoradiographed.

**Western Analysis**

Proteins were isolated from young and old livers and analyzed by Western blotting as described (4, 16). Inhibitors of phosphatases were included in all buffers used for the isolation of proteins or protein-protein complexes. Proteins were detected with antibodies against C/EBPβ (C19; Santa Cruz Biotechnology), CUGBP1 (monoclonal Abs from Santa Cruz Biotechnology; polyclonal Abs are characterized in our paper (6)), initiation translation factors eIFα and eIFβ, Grp78 (Santa Cruz Biotechnology), and CRT (Sigma). Antibodies to eIF2γ were provided by J. Hershey (University of California, Davis). True-Blot secondary antibodies and IP beads from Ebioscience were used for Western blotting and co-immunoprecipitation studies. The membranes were re-probed with antibodies to β-actin and stained with Coomassie. Protein levels of CUGBP1 were calculated as ratios of CUGBP1 signals to signals of β-actin by densitometry. Expression of C/EBPβ isoforms was calculated as a ratio of the isoforms to β-actin or to a cross-reactive molecule (50 kDa), which is usually detected with C19 antibodies (6). LIP/LAP ratio was calculated by densitometry as ratios of signals of LIP to signals of LAP after the subtraction of background.

**Gel Shift Analysis**

Conditions for electrophoretic mobility shift assay were described in our previous publication (16). Proteins (5–25 μg) were incubated with the uORF or ORF-LAP probes labeled with [γ-32P]ATP and T4 kinase.

**Purification of CUGBP1-LPPC from Old Livers**

The CUGBP1-LPPC complex was separated from free CUGBP1 by the size exclusion chromatography on SEC400 column (DuolFlow; Bio-Rad) as described previously (4, 15). High molecular weight fractions from three HPLC runs were combined and loaded on protein A-CUGBP1Ab column. Preliminary experiments were performed to find conditions under which the complex is eluted from the column without IgGs. We found that the optimal elution of the CUGBP1-eIF2 complex from the column is achieved by a gradient 0–0.1% SDS, Tris-HCl, pH 7.5. Under these conditions, CUGBP1 and IgGs remain linked to the column, whereas the complex is almost completely eluted in the fractions containing low concentrations of SDS. The eluted proteins were concentrated and loaded on denaturing SDS 8–16% gradient PAGE (Bio-Rad). Proteins were cut out and sequenced by mass spectrometry at the Baylor College of Medicine Core Facilities. As a control for the nonspecific absorption, the protein A column was pre-incubated with immune serum, and the HPLC-enriched CUGBP1-LPPC was loaded on this column. The isolation of the complex for examination of RNA associated with the complex was performed in the presence of RNase inhibitors. RNA was extracted from the complex and reverse-transcribed. The presence of C/EBPβ mRNA was determined by PCR with specific primers. The sequences of PCR primers are as follows: forward, 5′-CGGTCATGCAACGGCCTGTGGTGGGCC-3′; reverse, 5′-GAACGGGAAACCGGGCCGACTATGG-3′. Primers for liver-specific mRNA, PEPC, were used as a negative control for the nonspecific absorption of mRNAs. The sequences of these primers are as follows: PEPC-F, 5′-TTGGGACCGGATATGTTGGGGAC-3′; PEPC-R, 5′-TTTGTCTTCATGAGGTGCC-3′.

**Purification of Biologically Active CUGBP1-eIF2 by a Combination of HPLC-based Chromatography**

We have performed three steps of purification of the CUGBP1-eIF2 complex, which were sufficient to isolate the complex close to homogeneity and prevent the loss of its bio-
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logical activity. A strategy of the isolation is shown in Fig. 3B. Cytoplasmic proteins from old livers were fractionated by two steps on ion exchange UnoQ column. The first step was performed by elution of the complex with 0.05–0.5 M NaCl. The location of the complex in ion exchange fractions was determined by gel shift with the ORF-LAP probe. Fractions with the CUGBP1-eIF2 complex were combined, and NaCl within fractions was diluted to 0.1 M, and the complex was re-loaded on the UnoQ column. The second elution was performed with gradient 0.15–0.3 M NaCl. The complex was further loaded on the CHT1 column and eluted by a gradient of sodium phosphate buffer. The location of the complex was monitored by gel shift with ORF-LAP probe. After elution from CHT1 column, the complex was concentrated and loaded on 8–16% PAGE containing 0.1% SDS. The identity of proteins within the purified complex was determined by mass spectrometry sequencing and by Western blotting with antibodies to CUGBP1, eIF2α, β and γ, CRT, eR99, Grp78, and eR60.

Generation of uORF C/EBPβ Mutant and Translation of C/EBPβ mRNA in Reticulocyte Lysate (RL)

To examine the effects of CUGBP1-eIF2 on translation of C/EBPβ mRNA, we have generated a construct that contains a mutation of the AUG codon of uORF (uORFmut, ATG-ATT). A cell-free coupled transcription/translation system in rabbit reticulocyte lysate was used to examine translation of C/EBPβ and the effects of the CUGBP1-eIF2 complex on the translation. A purified CUGBP1-eIF2 complex was added into translation mixtures programmed with WT and the mutant C/EBPβ mRNAs in the presence [35S]methionine (Amersham Biosciences). After translation, C/EBPβ was immunoprecipitated from the reaction mixtures with specific antibodies (C-19; Santa Cruz Biotechnology) and analyzed by electrophoresis and autoradiography.

Examination of Translation of C/EBPβ in Cultured Cells

FLAG-tagged C/EBPβ constructs were co-transfected with GFP-CUGBP1 into Hep3B2 or in HEK293 cells. The formation of the GFP-CUGBP1-eIF2 complex was examined by co-IP approach. Under conditions of our experiments, the transcription of C/EBPβ mRNA from the WT and mutant construct was identical (data not shown). Translation of protein isoforms of C/EBPβ from these mRNA was examined by Western blotting with antibodies to the FLAG tag. A summary of multiple experiments in Hep3B2 and HEK293 cells is presented in this paper.

Analysis of the Role of CUGBP1 in the Formation of CUGBP1-eIF2 Complex

Mouse C2C12 cells were transfected with siRNA to inhibit expression of CUGBP1 as described in our previous paper (17). Cytoplasmic and nuclear extracts were isolated and used for examination of CUGBP1 levels (Western blotting) and for detection of CUGBP1-eIF2 complex by gel shift with ORF-LAP probe.

Examination of the CUGBP1-eIF2 Complex in CUGBP1 Transgenic Mice and in Young Mice Overexpressing Cyclin D3

CUGBP1-transgenic Mice—Generation and characterization of CUGBP1 transgenic mice are described in our previous paper (17). Protein extracts were isolated as described above, and CUGBP1-eIF2 complex was examined by co-IP and gel shift assays.

Injection of Cyclin D3 into Young Mice—We have delivered a cyclin D3 coding plasmid, pAdTrack-cyclin D3, to the liver of young (4 months) mice by the in vivo-jetPEI transfection reagent. Control animals were injected with an empty vector. The expression of cyclin D3 was examined in livers of control mice and in mice injected with pAdTrack-cyclin D3 plasmid by Western blotting. We have reproducibly observed a 3–5-fold induction of cyclin D3 in animals injected with the cyclin D3 plasmid. Animals with higher levels of cyclin D3 were used for studies presented in this study. The formation of the CUGBP1-eIF2 complex was determined by co-IP and gel filtration assays. This study represents a summary of data obtained in three control animals and in three mice injected with cyclin D3 plasmid.

RESULTS

Increased Expression of C/EBPβ Isoforms in Livers of Old Mice Correlates with Elevation of Protein Levels of CUGBP1—Levels of LIP are increased in old livers and might contribute to the age-associated alterations in biological processes such as liver proliferation (8, 18). We have begun investigations of age-mediated molecular mechanisms of translation of LIP in the liver. We have initially examined protein levels of C/EBPβ isoforms in mice used for these studies. Western blotting analysis of C/EBPβ expression in four animals of each age group is shown in Fig. 1A. Consistent with previous reports, expression of LIP is increased in old livers. In addition, we have observed a 2-fold induction of the LAP C/EBPβ isoform. Because the LIP/LAP ratio is critical for the ability of LIP to inhibit full-length C/EBP proteins (10), we calculated this ratio in young and old livers. In young animals this ratio is 0.3, whereas the LAP ratio in old livers is significantly increased and reaches 1.2 (Fig. 1B). These high levels of LIP might block activities of the full-length C/EBPβ, suggesting that LIP might interfere with activities of full-length C/EBP proteins in old livers.

Because CUGBP1 increases translation of LIP in proliferating livers and in cultured cells (5, 6, 16), we next examined if CUGBP1 might be involved in the translational induction of LIP in old animals. Western blotting with polyclonal Abs to CUGBP1 showed that CUGBP1 levels are increased 2-fold in old livers (Fig. 1C). Western blotting with monoclonal Abs to CUGBP1 confirmed these data (Fig. 1C, bottom). To examine the ability of CUGBP1 from old livers to regulate LIP translation, CUGBP1 was immunoprecipitated from young and old livers and added to a cell-free translation system programmed with WT C/EBPβ mRNA and with ATG3 mut C/EBPβ mRNA in which the third AUG codon is mutated. This mutant serves as a control for the possible production of LIP through specific proteolytic cleavage (19). As can be seen in Fig. 1D, CUGBP1 IPs from old livers significantly increase translation of LIP and slightly increase translation of FL and LAP from WT C/EBPβ mRNA. The increase of LIP translation is specific, because the addition of CUGBP1 IPs to the ATG3 mutant C/EBPβ mRNA increases translation of FL and LAP only. To examine if the CUGBP1-mediated increase of translation is specific to C/EBPβ mRNA and does not affect total translation, we have
tested the effects of CUGBP1 IPs on the translation of mRNA coding for the upstream stimulatory factor (16). As one can see in Fig. 1D, translation of upstream stimulatory factor is not affected by CUGBP1 IPs from either young or old livers. Thus, these studies revealed that old livers activate CUGBP1 and that the active CUGBP1 is able to specifically increase translation of all isoforms of C/EBPβ.

Old Livers Contain a Large CUGBP1 Protein-Protein Complex That Binds to the 5′ Region of C/EBPβ mRNA—We next determined if the CUGBP1 in old livers binds to the 5′ region of C/EBPβ mRNA. Previous investigations mapped a sequence within the 5′ region of C/EBPβ mRNA, from the first to the second AUG codons (Fig. 2A, LAP-ORF sequence), which is responsible for the CUGBP1-mediated induction of LIP translation. Gel shift experiments with the LAP-ORF probe revealed a dramatic difference in the patterns of binding between young and old livers. In young livers, two major RNA protein complexes interact with the LAP-ORF probe. However, only one major RNA-binding complex, which is located close to the top of the gel, is observed in old livers (Fig. 2B). To examine if this LPPC contains CUGBP1, we fractionated cytoplasmic proteins from old livers by ion exchange chromatography using an UNOQ column (Bio-Rad), and we performed a combined gel shift-Western blot assay as shown in Fig. 2C. A direct Western blotting with antibodies to CUGBP1 showed that CUGBP1 is distributed throughout the fractions (Fig. 2C, upper panel). Gel shift analysis with the LAP-ORF probe detected the LPPC within fractions eluted with 0.1–0.2 M NaCl. The upper part of the gel, which contains the LPPC, was cut out and loaded on the preparative SDS gradient 8–16% PAGE. Proteins were transferred on nitrocellulose and probed with monoclonal Abs to CUGBP1. These studies show that CUGBP1 is observed within the LPPC. To confirm this result, we have performed Western blotting with polyclonal Abs to CUGBP1. As one can see, polyclonal antibodies to CUGBP1 also detect CUGBP1 within the LPPC. Thus, these studies demonstrate that the aging liver increases amounts of the large protein-protein complex, which contains CUGBP1 and interacts with the 5′ region of C/EBPβ mRNA.

CUGBP1-LPPC Complex Consists of Translation Initiation Factor and Proteins of the Endoplasmic Reticulum—We next purified the CUGBP1-LPPC complex and determined the identity of the protein components of this complex. Free CUGBP1 and CUGBP1-LPPC were separated by size exclusion chromatography as shown in Fig. 3A. The age-specific CUGBP1-LPPC is located in fractions with molecular masses ranging from 800 kDa to void volume (>1 million). The fractions with CUGBP1-LPPC (three HPLC runs combined) were collected and loaded onto a protein A-anti-CUGBP1 column. The proteins associated with CUGBP1 were then removed from the column by a gradient of 0.0–0.1% SDS, the conditions under which CUGBP1 and IgGs remain on the protein A column. Electrophoretic analysis showed eight proteins that were detected by Coomassie stain. A parallel experiment with a protein A column loaded with IgGs from preimmune serum (agarose) showed that two proteins (identified by mass spectrometry as serum albumin and clathrin heavy chain) are attached to the protein A-anti-CUGBP1 column. The proteins were eluted with 0.1–0.2 M NaCl. The upper part of the gel, which contains the LPPC, was cut out and loaded on the preparative SDS gradient 8–16% PAGE. Proteins were transferred on nitrocellulose and probed with monoclonal Abs to CUGBP1. These studies show that CUGBP1 is observed within the LPPC. To confirm this result, we have performed Western blotting with polyclonal Abs to CUGBP1. As one can see, polyclonal antibodies to CUGBP1 also detect CUGBP1 within the LPPC. Thus, these studies demonstrate that the aging liver increases amounts of the large protein-protein complex, which contains CUGBP1 and interacts with the 5′ region of C/EBPβ mRNA.
column by nonspecific absorption. We conclude that the other six proteins and CUGBP1 are components of the CUGBP1-LPPC complex. The identity of these proteins was determined by mass spectrometry and confirmed by Western blot analyses. The CUGBP1-LPPC contains endoplasmic reticulum proteins eR99, CRT, eR60, Grp78, and two subunits of the eukaryotic initiation factor eIF2. All these proteins were reproducibly observed in three independent isolates of CUGBP1-LPPC from three animals. Although the biologically active eIF2 complex consists of three subunits, eIF2α, eIF2β, and eIF2γ, the eIF2γ subunit was not detectable within the CUGBP1-LPPC complex isolated by the immunopurification approach. We suggest that the eIF2γ subunit might be lost during the isolation. Note that CUGBP1 was also not detected in these samples because it remained on the beads. The presence of α and β subunits of the initiation factor eIF2 in the CUGBP1-LPPC (further called as CUGBP1-eIF2) complex suggested that this complex might be involved in the regulation of translation.

**Isolation of Biologically Active CUGBP1-eIF2 Complex from Old Livers**—To purify the biologically active CUGBP1-eIF2 complex, we performed fractionations using chromatography columns UnoQ and CHT1 as shown in Fig. 3B. The examination of CUGBP1-eIF2 by Coomassie stain after the last step (CHT1 column) showed that the polypeptide composition of the complex was close to the composition of the complex purified by immunoprecipitation (data not shown). To determine the identity of the proteins within this complex, we have sequenced individual polypeptides and performed Western blotting with antibodies to CUGBP1, Grp78, eIF2α, and CRT. As can be seen in Fig. 3C, all of these proteins are observed within the isolated CUGBP1-eIF2 complex. In the course of these studies, we have observed that two proteins, CUGBP1 and CRT, possess a retarded electrophoretic mobility within the complex and migrate in the positions that correspond to those of hyper-phosphorylated forms. As can be seen in Fig. 3C, the hyper-phosphorylated forms of CUGBP1 and CRT represent

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**FIGURE 2.** Aging increases the amount of a large CUGBP1-protein-protein complex that binds to the 5' region of C/EBPβ mRNA. A, nucleotide sequences of the 5' region of C/EBPβ mRNA. Positions of AUG codons for the full-length, LAP, and AUG of uORF are shown on the top. B, gel shift analysis of young and old cytoplasm with LAP-ORF probe. Positions of complexes with free CUGBP1 and CUGBP1-LPPC are shown on the right. C, cytoplasmic large protein-protein complex contains CUGBP1. Cytoplasmic proteins from old livers were separated on ion exchange UnoQ column. Upper, Western blotting shows examination of CUGBP1 within the UnoQ fractions. Gel shift demonstrates examination of UnoQ fractions by gel shift assay with the LAP-ORF probe. Upper region of the gel was cut out and loaded on SDS-gel electrophoresis. Bottom part, gel shift, Western blot, shows examination of CUGBP1 after separation of the proteins located on the top of the gel shift by SDS electrophoresis. Monoclonal and polyclonal Abs to CUGBP1 showed similar results.
minor fractions of the total proteins, but they are the major forms within the age-specific complex. The treatment of the CUGBP1-eIF2 complex with CIP shifted CRT to the position of unphosphorylated protein (Fig. 3D). Given the observations that the phosphorylated form of CUGBP1 is detected within the CUGBP1-eIF2 complex from high molecular weight regions of the gel filtration, right part shows electrophoretic analysis of the purified complex and identification of the proteins that was determined by mass spectrometry. Ag, control immunoprecipitation of the proteins from high molecular weight fractions with agarose loaded with a preimmune serum. Two proteins, clathrin heavy chain (CLTC) and serum albumin (SA), were absorbed to the column through nonspecific interactions. B, diagram showing a strategy for the isolation of CUGBP1-eIF2 complex by combination of HPLC. C, Western blotting examination of the complex with antibodies to the components of the complex. Tot, total cytoplasmic proteins (50 μg/lane); Comp, CUGBP1-eIF2 complex. D, CRT is hyper-phosphorylated within the CUGBP1-eIF2 complex. The purified complex was treated with CIP and examined by Western blotting with antibodies to CRT. E, phosphorylation of CUGBP1 is increased in cytoplasm of old livers. CUGBP1 was immunoprecipitated from cytoplasm of young and old livers, separated by two-dimensional gel electrophoresis and probed with monoclonal antibodies to CUGBP1. Bottom part shows examination of CUGBP1 after treatment of IPs with CIP. Positions of age-specific isoforms of CUGBP1 are shown by red arrows.

FIGURE 3. Isolation of CUGBP1-LPPC from old livers. A, immuno-isolation of CUGBP1-LPPC. Gel shift (upper image) shows examination of CUGBP1-LPPC within size exclusion fractions by gel shift with the LAP-ORF probe. Positions of HPLC markers are shown on the top. A diagram on the left shows immuno-purification of the CUGBP1-LPPC complex from high molecular weight regions of the gel filtration. Right part shows electrophoretic analysis of the purified complex and identity of the proteins that was determined by mass spectrometry. Ag, control immunoprecipitation of the proteins from high molecular weight fractions with agarose loaded with a preimmune serum. Two proteins, clathrin heavy chain (CLTC) and serum albumin (SA), were absorbed to the column through nonspecific interactions. B, diagram showing a strategy for the isolation of CUGBP1-eIF2 complex by combination of HPLC. C, Western blotting examination of the complex with antibodies to the components of the complex. Tot, total cytoplasmic proteins (50 μg/lane); Comp, CUGBP1-eIF2 complex. D, CRT is hyper-phosphorylated within the CUGBP1-eIF2 complex. The purified complex was treated with CIP and examined by Western blotting with antibodies to CRT. E, phosphorylation of CUGBP1 is increased in cytoplasm of old livers. CUGBP1 was immunoprecipitated from cytoplasm of young and old livers, separated by two-dimensional gel electrophoresis and probed with monoclonal antibodies to CUGBP1. Bottom part shows examination of CUGBP1 after treatment of IPs with CIP. Positions of age-specific isoforms of CUGBP1 are shown by red arrows.

minor fractions of the total proteins, but they are the major forms within the age-specific complex. The treatment of the CUGBP1-eIF2 complex with CIP shifted CRT to the position of unphosphorylated protein (Fig. 3D). Given the observations that the phosphorylated form of CUGBP1 is detected within the CUGBP1-eIF2 complex, we have next compared phosphorylation of CUGBP1 in young and old livers using a two-dimensional technique. CUGBP1 was immunoprecipitated from the cytoplasm of young and old livers with polyclonal Abs, separated by two-dimensional gel electrophoresis and probed with antibodies to CUGBP1. As one can see in Fig. 3E, two additional isoforms of CUGBP1 are observed in old livers compared with the isoforms seen in young livers. These isoforms are located in the acidic region of the two-dimensional gel, and the treatment of the extracts from old livers with alkaline phosphatase shifted these isoforms to the alkaline region. These data showed that the phosphorylation of CUGBP1 is increased in old livers and that phosphorylated form of CUGBP1 is the major isoform within the age-specific CUGBP1-eIF2 complex. Taken together, the analysis of the CUGBP1-eIF2 complex purified from the cytoplasm of old livers under native conditions confirmed the composition of the complex and revealed that at least two components (CUGBP1 and CRT) are hyper-phosphorylated within the complex.

CUGBP1 Is a Key Component of the CUGBP1-eIF2 Complex—We have examined if the CUGBP1-eIF2 complex is associated with C/EBPβ mRNA in old livers. The CUGBP1-eIF2 complex was isolated from the cytoplasm of old livers by an immunoprecipitation procedure as shown in Fig. 3A. RNA was isolated from the complex and examined in RT-PCR with primers to C/EBPβ mRNA. Primers to PEPCK mRNA were used as a negative control. As can be seen in Fig. 4A, the complex is associated with C/EBPβ mRNA in old livers, whereas PEPCK mRNA is not detected within the complex. Because the complex contains
eight proteins, we have next examined the RNA binding activity of the purified complex. For this goal, an UV cross-linking assay was performed with the LAP-ORF probe. This assay showed that the complex contains three proteins that bind to C/EBPβ mRNA. One of these proteins is CUGBP1, because it can be immunoprecipitated from UV cross-linking reactions with specific Abs (Fig. 4B). The identity of the two other proteins that interact with the ORF-LAP probe is unknown. Although CRT is able to bind to the 5' region of C/EBPβ mRNA (16), we could not detect the interaction of CRT within the CUGBP1-eIF2 complex with the LAP-ORF probe. These data are consistent with our previous results showing that only a small portion of CRT (2–3%) is able to interact with RNA (4). To determine the role of CUGBP1 in the formation of the complex, we inhibited CUGBP1 by siRNA in C2C12 cells and examined the CUGBP1-eIF2 complex. These C2C12 cells were selected for the studies because the CUGBP1-eIF2 complex is abundant in these cells. Results of these studies are shown in Fig. 4C. The expression of CUGBP1-specific siRNA causes a 3–4-fold reduction of CUGBP1. A parallel examination of the binding activity of the CUGBP1-eIF2 complex by gel shift showed that the inhibition of CUGBP1 significantly reduces the amount of CUGBP1-eIF2 in the cytoplasm. These studies demonstrated that CUGBP1 plays a critical role in the formation of the CUGBP1-eIF2 complex. Because hyper-phosphorylated forms of CUGBP1 are the major isoforms of CUGBP1 observed within the complex, we determined whether phosphorylation of CUGBP1 is involved in the age-associated formation of the CUGBP1-eIF2 complex by examining the effects of alkaline phosphatase on the complex. Cytoplasmic extracts from young and old livers, and extracts from old livers treated with CIP, were separated by size exclusion chromatography on SEC-400 column. The locations of CUGBP1 and eIF2 within fractions were examined by Western blotting with antibodies to CUGBP1 and to eIF2α. The CUGBP1-eIF2 complex was identified by co-immunoprecipitation assay: CUGBP1-IP, eIF2α Western.

![Image](86x626 to 190x685)

**FIGURE 4.** The role of CUGBP1 in biological functions of the CUGBP1-eIF2 complex. A, CUGBP1-eIF2 complex is associated with C/EBPβ mRNA in old livers. CUGBP1-eIF2 complex was purified by the immuno-isolation approach as shown in Fig. 3A. RNA was purified from the complex and reverse-transcribed. PCR was performed with primers specific to C/EBPβ mRNA and to PEPCK mRNA. Control, RT-PCR with total RNA from liver. H2O, a negative control. Positions of PCR products for C/EBPβ and PEPCK are shown by arrowheads. B, CUGBP1 is a component of the complex that binds to the 5' region of C/EBPβ mRNA. Two fractions of the CHT1 isolation of the complex (labeled on the top as 1 and 2) were incubated with LAP-ORF probe, linked by UV treatment, and separated by gel electrophoresis. 3rd lane was loaded with CUGBP1 IP after UV cross-linking the CUGBP1-eIF complex (longer exposure of this lane is shown). Positions of CUGBP1 and two other RNA-binding proteins are shown on the right. C, CUGBP1 is required for the formation of the CUGBP1-eIF2 complex. C2C12 myocytes were transfected with siRNA to CUGBP1. Cytoplasmic and nuclear extracts were isolated and examined for the expression of CUGBP1 (Western blot) and for the binding activity of the CUGBP1-eIF2 complex (gel shift). After exposure, the CUGBP1 membrane was re-probed with antibodies to β-actin to verify protein loading. D, de-phosphorylation of cytoplasmic proteins destroys the CUGBP1-eIF2 complex. Cytoplasm from young and old mouse livers, and cytoplasm of old livers treated with CIP, was separated by size exclusion chromatography on SEC-400 column. The locations of CUGBP1 and eIF2 within fractions were examined by Western blotting with antibodies to CUGBP1 and to eIF2α. The CUGBP1-eIF2 complex was identified by co-immunoprecipitation assay: CUGBP1-IP, eIF2α Western.
gested that phosphorylation of the components of the CUGBP1-eIF2 complex is important for the formation of the complex in old livers.

**Elevation of CUGBP1 in Young Livers Increases Amounts of CUGBP1-eIF2 Complex**—Given a correlation between the elevation of CUGBP1 in old livers and the appearance of the CUGBP1-eIF2 complex, we next examined if the overexpression of CUGBP1 in young livers is sufficient for the increase of the CUGBP1-eIF2 complex and if the complex regulates translation of C/EBPβ. For this goal, we have used CUGBP1 transgenic mice, in which his-CUGBP1 was placed under a modified β-actin promoter directing expression of his-CUGBP1 in muscle, heart, and to a lesser extent in liver (17). Because expression of CUGBP1 is age-dependent (Fig. 1), we first compared protein levels of CUGBP1 in livers of WT and CUGBP1 transgenic mice of different ages. Cytoplasmic extracts from WT and CUGBP1 transgenic mice of 6-, 12-, and 24-month-old animals were examined by Western blotting with antibodies to CUGBP1. The membrane was stripped and re-probed with β-actin. C2C12, cytoplasmic proteins from mouse myocytes C2C12 serve as a positive control. Bottom part shows a summary of five independent experiments. Protein levels of CUGBP1 were calculated as ratios to β-actin and then normalized to levels observed in 6-month-old wild type livers. B, examination of the CUGBP1-eIF2 complex in 12-month-old CUGBP1 transgenic mice by co-IP. CUGBP1 was immunoprecipitated from 12-month-old WT and CUGBP1 transgenic livers (LTR) with monoclonal antibodies to CUGBP1. CUGBP1 IPs were probed with antibodies to elf2α, elf2β, and CRT. Bottom part (IgG) shows heavy chains of antibodies detected by Western blotting. C, RNA binding activity of the CUGBP1-eIF2 complex is increased in CUGBP1 transgenic livers. Gel shift of cytoplasmic extracts was performed with the LAP-ORF probe. D, amounts of LAP and LIP are increased in livers of CUGBP1 transgenic mice. Cytoplasmic (for his-CUGBP1) and nuclear extracts (for C/EBPβ) from 12-month-old wild type (WT) and CUGBP1 transgenic livers (LTR) were examined by Western blotting with antibodies to CUGBP1 (upper) and to C terminus of C/EBPβ (bottom). Bar graphs, levels of C/EBPβ isoforms were calculated as LIP/LAP ratios. E, CUGBP1-eIF2 complex from 12-month-old CUGBP1 transgenic mice increases translation of LAP and LIP in a cell-free translation system. CUGBP1-eIF2 complex was immunoprecipitated with monoclonal antibodies to CUGBP1 from WT and CUGBP1 transgenic livers and added into reticulocyte lysate programmed with WT and ATG3 mutant C/EBPβ mRNAs. A short exposure for LAP is shown on the top.
CUGBP1-eIF2 Complex in Aging Liver

At the age of 24 months, the levels of CUGBP1 do not differ significantly in WT and CUGBP1 transgenic livers. We next examined the formation of the CUGBP1-eIF2 complex in the livers of wild type and CUGBP1 transgenic mice of age 6, 12, and 24 months by examining eIF2α in CUGBP1 IPs. The CUGBP1-eIF2 complex is not detectable in 6- and 12-month-old wild type animals and is observed only in 24-month-old animals, whereas in CUGBP1-TR animals the complex is detected at 12 months (data not shown and see Fig. 5B). Therefore, we next performed a detailed examination of the CUGBP1-eIF2 complex in 12-month-old CUGBP1-TR mice by several approaches. CUGBP1 was immunoprecipitated from wild type and CUGBP1 transgenic livers, and these IPs were examined by Western blotting with antibodies to the components of the complex. As can be seen in Fig. 5B, the components of the CUGBP1-eIF2 complex are observed in CUGBP1 IPs. Similar experiments with CUGBP1-IPs from WT livers did not detect the complex. Gel shift analysis with cytoplasmic extracts from WT and CUGBP1 transgenic livers of 12-month-old animals showed that the binding activity of the CUGBP1-eIF2 complex toward C/EBPβ mRNA is increased in livers of CUGBP1 transgenic mice (Fig. 5C). Taken together, these studies show that the amount of the CUGBP1-eIF2 complex is increased in 12-month-old CUGBP1 transgenic livers, mimicking the induction of the CUGBP1-eIF2 complex in wild type 24-month-old livers.

His-CUGBP1-eIF2 Complex Increases Translation of LIP and LAP in the Liver—We next examined if translation of C/EBPβ isoforms is altered in CUGBP1-TR livers. Western blotting analysis of liver nuclear extracts from 12-month-old mice shows that expression of LIP is weak or not detectable in wild type mice, whereas LIP is expressed at high levels in livers of CUGBP1 transgenic animals (Fig. 5D). We have found that, similar to the expression of C/EBPβ in old livers, protein levels of LAP are also increased 2-fold in CUGBP1-TR livers. Calculations of the LIP/LAP ratio showed that the relative amounts of LIP are similar to those observed in old livers and are sufficient to compete with the biological functions of full-length C/EBP proteins (Fig. 5D, bar graphs). We next examined the ability of the CUGBP1-eIF2 complex to increase translation of LIP using a cell-free translation system. The complex was immunoprecipitated with antibodies to CUGBP1, and the IPs were added into reticulocyte lysate programmed with WT and with ATG3 mutant C/EBPβ mRNAs. These studies showed that the CUGBP1-eIF2 complex significantly increases translation of LIP and, to a lesser extent, translation of LAP (Fig. 5E). The increase of LIP by CUGBP1 IPs is mediated through the translational mechanism because the ATG3 mutant C/EBPβ mRNA does not produce this isoform. Taken together, the investigations of CUGBP1 transgenic mice demonstrated that, similar to old livers, the elevation of CUGBP1 in young livers leads to the induction of the CUGBP1-eIF2 complex, which increases translation of both isoforms of C/EBPβ.

Cyclin D3-cdk4 Hyper-phosphorylates CUGBP1 in Old Livers and Increases Formation of the CUGBP1-eIF2 Complex—In addition to the elevation of protein levels of CUGBP1, old livers also have increased phosphorylation of CUGBP1 (Fig. 3E). Because the phosphorylated form of CUGBP1 is the major isoform within the CUGBP1-eIF2 complex, we have searched for possible kinases that are activated in old livers and might be involved in the appearance of the CUGBP1-eIF2 complex. We have recently observed that old livers increase activity of cyclin D3-cdk4 by the elevation of protein levels of cyclin D3 in nuclei (20). Therefore, we have examined whether this kinase is active in the cytoplasm of old livers (where the CUGBP1-eIF2 complex is located) and whether this kinase might phosphorylate CUGBP1. Western blotting showed that protein levels of cyclin D3 are increased in the cytoplasm of old livers. Calculations of cyclin D3 levels as ratios to β-actin revealed a 3–5-fold higher expression of cyclin D3 in the cytoplasm of old livers (Fig. 6A). Examination of kinase activities associated with cyclin D3 using retinoblastoma protein as substrate showed that cyclin D3-cdk4/6 is more active in the cytoplasm of old livers (Fig. 6B). To determine whether the elevation of cyclin D3 in the cytoplasm of old livers increases cdk4 kinase activity toward CUGBP1, cdk4 and CUGBP1 were immunoprecipitated from the cytoplasm and added into an in vitro kinase assay with bacterially expressed, purified MBP-CUGBP1. As can be seen in Fig. 6C, cdk4 IPs from old livers phosphorylate CUGBP1, whereas the phosphorylation of CUGBP1 by cdk4 IPs from young livers is much weaker. Interestingly, CUGBP1 IPs from old livers also display a kinase activity that phosphorylates bacterially expressed MBP-CUGBP1. This kinase appears much stronger in the cytoplasm of old livers than young. These observations suggest that CUGBP1 is associated with cdk4-cyclin D3 in the liver, and cdk4 co-immunoprecipitates with CUGBP1.

To further examine the role of cdk4 in the phosphorylation of CUGBP1, we have examined if the baculovirus expressed, purified cyclin D1-cdk4 complex phosphorylates CUGBP1 in vitro. As one can see in Fig. 6D (left), the purified cyclin D1-cdk4 phosphorylates CUGBP1. Because the phosphorylation of CUGBP1 by cdk4 was lower than phosphorylation of retinoblastoma protein, we examined if this phosphorylation is specific. For this goal, we have performed analysis of several CUGBP1 mutants, in which Ser/Thr residues were substituted by glycine. We have found that mutation of Ser-302 of CUGBP1 phosphorylates CUGBP1, we have examined if the baculovirus expressed, purified MBP-CUGBP1. As can be seen in Fig. 6D (left), the purified cyclin D1-cdk4 phosphorylates CUGBP1. Because the phosphorylation of CUGBP1 by cdk4 was lower than phosphorylation of retinoblastoma protein, we examined if this phosphorylation is specific. For this goal, we have performed analysis of several CUGBP1 mutants, in which Ser/Thr residues were substituted by glycine. We have found that mutation of Ser-302 of CUGBP1 significantly reduces the cdk4-mediated phosphorylation of CUGBP1 (Fig. 6D). Thus, these studies showed that cdk4 phosphorylates CUGBP1 in vitro and that Ser-302 is a key residue of CUGBP1 that is phosphorylated by cdk4.

To examine if cdk4 is the kinase that contributes to the elevation of the CUGBP1-eIF2 complex in old livers, we have overexpressed cyclin D3 in young livers by injecting a plasmid coding for cyclin D3 into the tail vein using in vivo jetPEI transfection reagent. Fig. 6E shows a typical result of Western blotting analysis of cyclin D3 levels in the liver of young animals (4 months) after injection of the pAdTrack-cyclin D3 plasmid and in control animals transfected with an empty vector. The calculations of the levels of cyclin D3 as ratios to β-actin showed a 4–5-fold increase in cyclin D3, which is close to the levels of cyclin D3 observed in old livers. Examination of cyclins A and E revealed that these kinases are not detectable in cytoplasmic extracts from livers of cyclin D3-injected mice (Fig. 6E, bottom), suggesting that the elevation of cyclin D3 is responsible for the increase of the CUGBP1-eIF2 complex (see below). The formation of the CUGBP1-eIF2 complex was examined in...
these animals by co-IP assay. CUGBP1 was immunoprecipitated with specific antibodies, and the components of the complex were examined by Western blotting with antibodies to cyclin D3. The membrane was re-probed with antibodies to β-actin, and the levels of cyclin D3 were calculated as ratios to β-actin (shown below). The membrane was stained with Coomassie. 

**Figure 6.** Activation of cdk4 by cyclin D3 leads to the phosphorylation of CUGBP1 and to the appearance of the CUGBP1-eIF2 complex in young mice. 

A, old livers increase expression of cyclin D3 in the cytoplasm. Cytoplasmic proteins from young and old livers were examined by Western blotting with antibodies to cyclin D3. The membrane was re-probed with antibodies to β-actin, and the levels of cyclin D3 were calculated as ratios to β-actin (shown below). The membrane was stained with Coomassie. 

B, elevation of cyclin D3 in cytoplasm of old livers increases activities of associated kinases. Cyclin D3 IPs from cytoplasm of young and old livers were examined in an in vitro kinase assay with retinoblastoma protein substrate. C, control cyclin D1-cdk4 kinase. C, kinase activity of cdk4 toward MBP-CUGBP1 substrate is increased in cytoplasm of old livers. Cdk4 and CUGBP1 were immunoprecipitated from cytoplasm of young and old livers and incubated with MBP-CUGBP1 in an in vitro kinase assay. Ag, mock control with agarose beads. The mixtures were loaded on the gel, transferred on the membrane, exposed with x-ray film, and stained with Coomassie to verify loading of sub-}

**Figure 6.** Activation of cdk4 by cyclin D3 leads to the phosphorylation of CUGBP1 and to the appearance of the CUGBP1-eIF2 complex in young mice. 

A, old livers increase expression of cyclin D3 in the cytoplasm. Cytoplasmic proteins from young and old livers were examined by Western blotting with antibodies to cyclin D3. The membrane was re-probed with antibodies to β-actin, and the levels of cyclin D3 were calculated as ratios to β-actin (shown below). The membrane was stained with Coomassie. 

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The CUGBP1-eIF2 Complex Increases Translation of C/EBPβ mRNA Independent from Initiation on the uORF AUG Codon—We next performed studies to examine the mechanisms by which the CUGBP1-eIF2 complex increased translation of C/EBPβ. Previous studies have shown that the initiation of translation on the AUG codon of the uORF of C/EBPβ mRNA is a critical event in the regulation of translation of LAP and LIP (12–14). Therefore, we have generated a mutant construct (uORFmut), in which the AUG codon of the uORF was mutated to AUU. WT and uORFmut C/EBPβ constructs were cloned under a T7 promoter for expression in a cell-free translation system and under a cytomegalovirus promoter for the expression of FL, LAP, and LIP isoforms. The CUGBP1-eIF2 complex was added into reticulocyte lysate programmed with WT and with uORF mutant C/EBPβ (uORFmut). These studies showed that the CUGBP1-eIF2 complex increased translation of all isoforms of C/EBPβ from the uORF mutant construct. The CUGBP1-eIF2 complex attached to protein-A beads and control IP with agarose were added into reticulocyte lysate programmed with WT and with uORF mutant C/EBPβ mRNAs. As can be seen in Fig. 7A, the complex was added to the reticulocyte lysate programmed with WT and ATG3 mutant C/EBPβ mRNAs.

Confirm results obtained in a cell-free translation system (Fig. 7B). With the uORF mutant C/EBPβ mRNA, the translation of both isoforms of C/EBPβ appears increased. These observations are consistent with data published by Johnson and co-workers (12). We next examined the effects of the CUGBP1-eIF2 complex on the translation of WT and uORFmut C/EBPβ mRNAs in reticulocyte lysate. We assumed that if the CUGBP1-eIF2 complex releases the inhibitory effects of the uORF, the complex should not increase translation of C/EBPβ from the uORF mutant construct. The CUGBP1-eIF2 complex attached to protein-A beads and control IP with agarose were added into reticulocyte lysate programmed with WT and with uORFmut C/EBPβ mRNAs (Fig. 7C), suggesting that the increase of C/EBPβ translation by the complex is independent of the uORF. Because previous studies showed that LIP might also be produced by proteolytic cleavage, we tested the effects of the CUGBP1-eIF2 complex on the ATG3 mutant C/EBPβ mRNA. Fig. 7C (right) shows that the CUGBP1-eIF2-dependent induction of LIP is mediated through a translational mechanism because the addition of the CUGBP1-eIF2 complex to the translation reactions with the ATG3 mutant does not increase translation of LIP, whereas production of FL and LAP isoforms is increased. Taken together, these studies showed that the age-specific CUGBP1-eIF2 complex increases translation of all isoforms of C/EBPβ and that this increase does not depend on the initiation on the uORF AUG.

CUGBP1-eIF2 Complex Increases Translation of C/EBPβ Isoforms in Cultured Cells—To examine the effects of the CUGBP1-eIF2 complex on the translation of C/EBPβ in cultured cells, FLAG-tagged WT and uORF mutant C/EBPβ were co-transfected with GFP-CUGBP1 into Hep3B2 and HEK293 cells. Co-IP studies showed that ectopic expression of GFP-CUGBP1 leads to an increased association of CUGBP1 with eIF2 (Fig. 8A), suggesting that other components of the CUGBP1-eIF2 are also within the complex. Because the size of GFP-CUGBP1 is larger than that of endogenous CUGBP1, Western blotting with antibodies to CUGBP1 provides the ability to calculate the ratio of GFP-CUGBP1 to endogenous protein. As can be seen in Fig. 8B, transfection of GFP-CUGBP1 plasmid leads to a 3–4-fold elevation of CUGBP1 protein above the level of endogenous CUGBP1. Under these conditions, GFP-CUGBP1 increases translation of both LAP and LIP isoforms.

**FIGURE 7.** CUGBP1-eIF2 complex increases translation of C/EBPβ mRNA independent from initiation on the uORF AUG codon. A, translation of WT (W) and uORF mutant (uORFm) C/EBPβ constructs in reticulocyte lysate. Positions of FL, LAP, and LIP isoforms are shown. Bar graph shows a densitometric calculation of FL, LAP, and LIP isoforms as percentage of signals of each isoform relative to the sum of signals. B, expression of C/EBPβ isoforms from WT and uORF mutant constructs in Hep3B2 cells. The middle section shows a light exposure for LAP isoform. The filter was re-probed with Abs to β-actin. Bar graph shows a ratio of LIP and LAP to β-actin. C, the CUGBP1-eIF2 complex increases translation of all isoforms of C/EBPβ. Left image, the CUGBP1-eIF2 complex was added into reticulocyte lysate programmed with WT and with uORF mutant C/EBPβ mRNAs. Ag, mock control (see text). Right image, the complex was added to the reticulocyte lysate programmed with WT and ATG3 mutant C/EBPβ mRNAs.
forms of C/EBPβ from both WT and uORF mutant constructs. Calculation of LIP and LAP as ratios to β-actin showed that GFP-CUGBP1 increases translation of LAP up to 10-fold (Fig. 8C). Translation of LIP seems to be increased by GFP-CUGBP1 to higher levels of up to 20-fold. However, given a strong expression of LAP, it is quite difficult to determine a precise ratio of LIP/LAP in these experiments. Thus, the studies in RL and in cultured cells revealed that GFP-CUGBP1 increases translation of C/EBPβ mRNA, perhaps via formation of the CUGBP1-eIF2 complex.

Because GFP-CUGBP1 also increases translation of C/EBPβ isoforms from uORF mutant C/EBPβ mRNA, we suggested that there is an additional event in the CUGBP1-mediated increase of translation. Therefore, we have examined if GFP-CUGBP1 might increase an association of C/EBPβ mRNA with polysomes. To examine this possibility, we have determined the amount of C/EBPβ mRNA in polysomal fractions from cells transfected with C/EBPβ mRNA and from cells in which C/EBPβ mRNA was co-transfected with GFP-CUGBP1. Polysomal (PS) fractions were isolated from transfected cells with primers to C/EBPβ mRNA. The RNA without reverse transcription (RT−) was used as a control for DNA contaminations. PS, total RNA was isolated from the polysomal fractions, and C/EBPβ mRNA was examined by RT-PCR with specific primers. Results with two preparations of PS RNA are shown.

FIGURE 8. CUGBP1-eIF2 complex increases translation of C/EBPβ isoforms in cultured cells by recruiting a large portion of C/EBPβ mRNA to ribosomes. A, GFP-CUGBP1 forms a complex with eIF2. HEK293 cells were transfected with GFP-CUGBP1 plasmid and with an empty vector. Protein extracts were isolated and probed with antibodies to CUGBP1 (upper image). CUGBP1 was immunoprecipitated from cytoplasmic extracts, and eIF2α and CUGBP1 were examined in these IPs (bottom, IP). B, overexpression of CUGBP1 in HEK293 cells increases translation of C/EBPβ isoforms. Increasing amounts of GFP-CUGBP1 were co-transfected with WT and uORF mutant C/EBPβ mRNA into HEK293 cells. Cytoplasmic extracts were probed with antibodies to CUGBP1 (upper image), and nuclear extracts were examined by Western blotting with FLAG antibodies. Antibodies to CUGBP1 interact with endogenous CUGBP1 (51 kDa) and with GFP-CUGBP1 (80 kDa). Endogenous CUGBP1 serves as a loading control. The membrane with C/EBPβ isoforms was re-probed with Abs to β-actin (bottom panel). C, levels of LAP and LIP isoforms determined as ratios to β-actin. D, GFP-CUGBP1 complex is observed on the polysomes. Polysomal fractions were isolated from HEK293 cells transfected with WT C/EBPβ alone and from cells in which C/EBPβ was co-transfected with GFP-CUGBP1. Half of the polysomal fraction was examined by Western blotting with antibodies to CUGBP1 and eIF2α. E, the CUGBP1-eIF2 complex recruits a larger portion of C/EBPβ mRNA to polysomes. Input shows RT-PCR of total RNA isolated from transfected cells with primers to C/EBPβ mRNA. The RNA without reverse transcription (RT−) was used as a control for DNA contaminations.
**CUGBP1-eIF2 Complex in Aging Liver**

**DISCUSSION**

Aging Increases Amounts of the CUGBP1-eIF2 Complex in Liver—Our previous studies demonstrated a critical role of CUGBP1 in the translation of p21 during replicative senescence. In this system, the phosphorylation of CUGBP1 is a major pathway of activation of CUGBP1 (4). A detailed examination of the activation of CUGBP1 in old livers showed that aging activates CUGBP1 by phosphorylation and by the increase of protein levels of CUGBP1. Phosphorylated CUGBP1 is the major isoform that interacts with translation initiation factor eIF2 and forms the large age-specific CUGBP1-eIF2 complex (see Fig. 3). We have found that the specificity of the CUGBP1 interaction with C/EBPβ mRNA is altered within the age-specific complex. The CUGBP1-eIF2 complex binds preferentially to a probe covering the long 5’ region of C/EBPβ mRNA and has a weak interaction with a short uORF fragment, whereas homogeneous CUGBP1 identically interacts with both probes (data not shown). This observation suggests that the complex likely recognizes a secondary structure of the 5’ region of C/EBPβ mRNA. Biochemical purification of the CUGBP1-eIF2 complex from old livers revealed the presence of four proteins from the endoplasmic reticulum: eR99, Grp78, eR60, and calreticulin. The identification of CRT within the CUGBP1-eIF2 complex was surprising, because our previous studies showed that CRT competes with CUGBP1 for the regulation of translational targets through the direct interaction with mRNAs. However, within the CUGBP1-eIF2 complex, CRT does not bind to C/EBPβ mRNA. Consistent with the lack of RNA binding activity of CRT within the complex, the major form of CRT within the complex is a modified CRT that represents a minor portion of the total CRT (Fig. 3). The role of proteins of the endoplasmic reticulum in activities of the CUGBP1-eIF2 complex is not known. One possible scenario is that the phosphorylated CRT and the other proteins of the endoplasmic reticulum (eR99, Grp78, eR60) might be involved in guiding the CUGBP1-eIF2 complex to the endoplasmic reticulum and also in directing the translational products to certain cellular compartments.

The interaction of CUGBP1 with eIF2α seems to be a critical step in the regulation of the CUGBP1-eIF2 complex and perhaps requires the presence of three subunits of eIF2, -α, -β, and -γ. Under our experimental conditions, the γ subunit of the eIF2 complex was not detectable by Western blotting within the CUGBP1-eIF2 complex; however, indirect observations suggest that eIF2 within the CUGBP1 complex contains all three subunits. For example, size exclusion chromatography of the CUGBP1-eIF2 complex in CUGBP1 transgenic mice showed very good co-localizations of α, β, and γ subunits of eIF2 with other components of the complex (data not shown). In addition, the ability of the CUGBP1-eIF2 complex to increase translation suggests the presence of a functional eIF2 complex. Therefore, we suggest that the failure to detect the γ subunit of eIF2 in the CUGBP1 complex reflects a low sensitivity of our assays and/or a partial loss of the γ subunit during isolation of the complex.

**The Role of Cyclin D3-cdk4-mediated Phosphorylation of CUGBP1 in the Appearance of the CUGBP1-eIF2 Complex in Old Livers**—Aging changes activities of CUGBP1 by two pathways as follows: by the elevation of protein levels of CUGBP1 and by an increase of the phosphorylation of CUGBP1 (Figs. 1 and 3). We have identified a kinase that phosphorylates CUGBP1 in old liver and in vitro. Our data show that aging liver increases activity of cdk4 in the cytoplasm by elevation of cyclin D3 and that cdk4 phosphorylates CUGBP1 in an in vitro kinase assay. To further determine the contributions of the elevation of CUGBP1 and the contributions of cdk4-mediated phosphorylation to the age-associated increase of the CUGBP1-eIF2 complex, we have utilized the following two animal models: CUGBP1-TR mice and young mice overexpressing cyclin D3 after tail vein injections. Studies with CUGBP1-TR mice demonstrated that the increase of CUGBP1 is an important step in the appearance of the complex and that 12-month-old CUGBP1-TR mice have detectable amounts of the CUGBP1-eIF2 complex, whereas the complex is not detectable in WT animals of the same age. The induction of the CUGBP1-eIF2 complex in transgenic mice enhances the ability of CUGBP1 to increase translation of C/EBPβ mRNA in vitro and correlates with the elevation of LAP and LIP isoforms. Interestingly, although protein levels of CUGBP1 in CUGBP1-TR mice of 6 and 12 months old are higher, the CUGBP1-eIF2 complex is formed only in 12-month-old animals and is not detected in 6-month-old transgenic mice. This observation clearly demonstrated that an additional event, perhaps phosphorylation of CUGBP1, is also required for the formation of the complex. Consistent with this suggestion, we have found that the activation of cdk4 by cyclin D3 in 4-month-old (young) animals is sufficient to increase the CUGBP1-eIF2 complex (Fig. 6E). Thus, these studies in different animal models suggest that both elevation of CUGBP1 and cyclin D3-cdk4-mediated phosphorylation of CUGBP1 are important for the age-associated appearance of the CUGBP1-eIF2 complex.

**The Role of C/EBPβ in Aging**—The initial goal of these studies was to examine the age-associated mechanisms of translation of LIP in the liver. In the course of these studies, we have found that the expression of LAP is also increased in old livers and that the CUGBP1-eIF2 complex increases translation of both isoforms in a cell-free translation system and in CUGBP1 transgenic mice. These new findings suggest that biological processes in old livers might also be affected by LAP and full-length C/EBPβ proteins. Consistent with this suggestion, Sebastian et al. (21) have recently found that full-length C/EBPβ is required for Ras-induced senescence. The authors showed that in senescent fibroblasts full-length C/EBPβ decreases expression of several E2F target genes through an association with their promoters. Because the proliferative response of old livers is significantly reduced with age, it would be interesting to examine if the elevation of LAP and LIP in livers of old mice contributes to the inhibition of proliferation. Although both isoforms of C/EBPβ are increased in old livers, the induction of LIP is higher than that of LAP, leading to a change in the LAP/LAP ratio. This change suggests that transcription of certain liver-specific genes (targets of C/EBPβ) might be repressed in old livers by the elevation of LIP. Consistent with this suggestion and with the observations that LIP inhibits transcription of PEPCK (22), the transcription of PEPCK mRNA is significantly reduced in old livers (23). Further studies are required to elucidate if the elevation of C/EBPβ isoforms in old livers changes...
transcription of the C/EBP targets as well as transcription of E2F targets.

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