Expression of a dominant negative 20-kDa isoform of C/EBP/Enhancer-binding protein (C/EBPβ), LIP, is increased in proliferating livers and in tumor cells. Two RNA-binding proteins, CUGBP1 and calreticulin, have been implicated in the translational regulation of C/EBPβ. In this paper, we present evidence showing several critical steps by which liver increases translation of LIP after partial hepatectomy. At early stages after partial hepatectomy, liver activates CUGBP1 by a hyperphosphorylation. The activated CUGBP1 binds to the 5’ region of C/EBPβ mRNA and replaces calreticulin, which partially represses translation of C/EBPβ in quiescent livers. The hyperphosphorylated CUGBP1 also interacts with the α and β subunits of initiation factor eIF2. Our data demonstrate that the interaction of CUGBP1 with the eIF2α enhances the association of CUGBP1 with ribosomes and correlates with increased translation of LIP in the liver after partial hepatectomy. Our data support the hypothesis that CUGBP1 increases translation of LIP by the interaction with the eIF2α subunit. This facilitates subsequent recruitment of larger numbers of ribosomes to initiate translation of LIP.

C/EBP family transcription factors regulate a variety of biological processes in different tissues (1). Two members of the C/EBP family, C/EBPα and C/EBPβ, are expressed at high levels in the liver and are involved in the regulation of liver growth and differentiation (2–4). A number of recent studies have demonstrated that the protein expression of C/EBPα and C/EBPβ is controlled on different levels, including transcription and translation of mRNAs and regulation of protein stability (5–7). Single mRNAs of C/EBPα and C/EBPβ produce different isoforms through the use of alternative translation start sites from several in-frame AUG codons (8–11). The truncated isoforms of C/EBPα and -β (30 kDa and LIP, respectively) lack one of activation domains and have reduced capacities to regulate transcription. However, these isoforms are able to dimerize with full-length C/EBP proteins and inhibit their transcriptional activities. Since expression of these isoforms is increased in many biological processes including breast tumors (12), the elucidation of mechanisms by which cells generate these isoforms may be helpful in the development of therapeutic approaches.

The truncated isoform of C/EBPβ, LIP, has been shown to function as a dominant negative molecule that heterodimerizes with full-length C/EBP proteins and blocks their activities (8). Experiments in cultured cells suggested that LIP might also have biological activities distinct from the full-length C/EBP proteins (12). Since both LAP and LIP isoforms are translated from the same mRNA, the elucidation of the role of each isoform in the liver proliferation is difficult. Luedde et al. (13) have recently performed studies using adenovirus-mediated delivery of LAP and LIP in the liver following initiation of proliferation by partial hepatectomy. These studies showed that LAP and LIP have different functions in liver proliferation. Overexpression of LAP led to a delayed S-phase entry of hepatocytes after partial hepatectomy (PH), whereas overexpression of LIP resulted in earlier induction of cyclin A, cyclin E, and proliferating cell nuclear antigen (13). Since LIP promotes liver proliferation, understanding the molecular mechanisms by which the liver increases LIP translation is very important. Several studies have been undertaken to elucidate the mechanism of LIP translation. It has been found that the 5’ region of C/EBPβ mRNA contains a short out-of-frame open reading frame (sORF; also known as an upstream open reading frame, or uORF), which is involved in the regulation of LIP translation (14, 15). A number of investigations showed that translation of LIP from the third AUG codon is mediated by a “leaky scanning” mechanism. This mechanism suggests that a portion of ribosomes initiates translation at the AUG codon of the sORF, synthesizes 9-amino acid peptide, reaches the stop codon of the sORF, and dissociates. Since there is a very close proximity of the stop codon of sORF and the initiating AUG codon of LAP, the ribosomes are not able to start translation of LAP and continue scanning until they meet the third (LIP-specific) AUG codon (14). Translational control of LIP production has been further confirmed by examination of effects of the eukaryotic translation initiation factor 2α (eIF2α) on C/EBPβ expression. Calkhoven et al. (15) showed that a high eIF2α activity shifts the ratio of LAP/LIP toward a more truncated isoform, LIP. Investigations of molecular mechanisms that control the initiation of translation on the sORF-specific AUG codon suggested that certain RNA-binding proteins might reg-
ulate this process by interacting with the sORF region of C/EBPβ mRNA (6, 16, 17). We have identified two RNA-binding proteins, CUGBP1 and CRT, which specifically bind to the sORF (6, 17). CRT has been shown to interact with GC-rich stem-loop structures and might regulate processing of corresponding RNAs (18–20). Although CUGBP1 and CRT interact with the same sequence of the 5′ region of C/EBPβ mRNA, the consequences of these interactions are different. CRT binds to the 5′ region of C/EBPβ mRNA and stabilizes a stem-loop (SL) structure, leading to the inhibition of translation of C/EBPβ (6). On the contrary, the interaction of CUGBP1 with the 5′ region of C/EBPβ mRNA increases translation of C/EBPβ (17).

In this paper, we examined the detailed molecular mechanisms by which liver increases translation of LIP after PH. Our data suggest the following pathway. In quiescent livers, the expression of LIP is limited by a negative effect of CRT, which is associated with the 5′ region of C/EBPβ mRNA. Partial hepatectomy activates CUGBP1 by phosphorylation, and the phosphorylated CUGBP1 interacts with the α and β subunits of eIF2. The CUGBP1-eIF2 complex binds to the 5′ region of C/EBPβ mRNA and replaces CRT leading to recruitment of a larger number of ribosomes to initiate translation of the sORF. These additional ribosomes skip the LAP-specific AUC codon and start initiation of LIP translation at the third AUC codon. As a result, more ribosomes meet the LIP-specific AUC codon, and the translation of LIP is increased in regenerating livers. Given growth promotion activity of LIP (13), these data present a novel pathway by which RNA-binding proteins contribute to liver proliferation.

MATERIALS AND METHODS

Liver Regeneration and LPS Treatment—Partial hepatectomy and LPS treatment were performed as described in our previous publications (16, 21). Procedures for the isolation of protein extracts were described in our earlier papers (6, 21).

Western Analysis—Protein extracts (50–100 μg) were separated by 4–20% polyacrylamide gradient gel electrophoresis and transferred onto a nitrocellulose membrane. C/EBPβ, CUGBP1, CRT, eIF2α, eIF2β, eIF2γ, and eIF4E antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in dilutions recommended in the manufacturer’s protocol. To verify protein loading, membranes were stripped and reprobed with monoclonal antibodies against β-actin (Sigma) (1:1,000) and stained with Coomassie Blue.

UV Cross-linking Assay—UV cross-linking was performed as described (6, 22). sORF riboprobe (see Fig. 1D and Ref. 16) was labeled with T4 kinase and [γ-32P]ATP. MBP-CUGBP1 (10 μg) or cytoplasmic proteins (20 μg) were incubated with radioactive riboprobe, linked to the probe by UV irradiation, separated by SDS-gel electrophoresis, transferred onto membrane, and exposed to x-ray film. After exposure, the membrane was stained with Coomassie Blue to verify protein loading.

Two-dimensional Gel Electrophoresis—Proteins (150–200 μg) were separated by isoelectric focusing using 3.0–10.0 strips followed by 5–20% gradient polyacrylamide gel electrophoresis (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane and probed with monoclonal antibodies against CUGBP1 (3B1; Santa Cruz Biotechnology).

Transfections—HeLa cells were used for the analysis of C/EBPβ regulation by RNA binding proteins. FLAG-tagged C/EBPβ construct (23) was co-transfected with His-CRT and GFP-CUGBP1 in different ratios. Proteins were isolated in 16 h after transfections and analyzed by Western blotting with antibodies to FLAG, His, and CUGBP1 as described above. For the analysis of intracellular localization of CUGBP1 and its RNA binding domains (RBDs), HeLa cells were transfected with GFP-CUGBP1, GFP-RBD1, and GFP-RBD3 using FuGene (Invitrogen). Subcellular fractionations and Western analysis were performed as described below.

Isolation of CUGBP1-interacting Proteins—MBP-CUGBP1 was induced in E. coli with isopropyl 1-thio-β-d-galactopyranoside and then loaded on amylose resin. Cytoplasmic proteins from HeLa cells (10 mg) were incubated with the amylose resin containing MBP-CUGBP1. The column was washed extensively with phosphate-buffered saline buffer, and CUGBP1-binding proteins were eluted with 0.5 M NaCl. Eluted proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. For protein sequencing, proteins were transferred onto polyvinylidene difluoride membrane and stained with Coomassie Blue. Protein bands were cut out from the membrane and sequenced in the Baylor College of Medicine Protein Sequencing Core.

Examination of RNA-Protein and Protein-Protein Complexes by Size Exclusion Chromatography—Cytoplasmic proteins from HeLa cells and from mouse liver were separated by HPLC chromatography (DuoFlow; Bio-Rad) using SEC-400 column. Size exclusion runs were performed with the buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 5 mM dithiothreitol. Size exclusion fractions were analyzed by Western blotting with antibodies to CUGBP1, eIF2α, and eIF2β. To detect protein-protein complexes, CUGBP1 was immunoprecipitated from each fraction, and the α and β subunits of eIF2 were examined in CUGBP1 immunoprecipitations. To examine the role of RNA in the formation of CUGBP1-eIF2α-eIF2β complexes, cytoplasmic proteins were pre-treated with RNase A prior to chromatography. The size exclusion fractions were examined as described above.

Isolation of Polysomes—A detailed procedure for the analysis of polysomal fractions is described in our previous paper (22). Briefly, cells were homogenized with polysomal buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 25% sucrose. Nuclei and mitochondria were spun down by centrifugation at 15,000 rpm for 45 min. Supernatant (cytoplasm) was treated with 1% Triton X-100 and 0.5% deoxycholate at 2°C and centrifuged through 1.0 M sucrose prepared in polysomal buffer. The pellet (which contained a mixture of polysomes and individual 40 and 60 S ribosomal subunits) was resuspended in polysomal buffer and used for Western blotting.

RESULTS

The Increase of LIP in Regenerating Mouse Livers Correlates with the Elevation of CUGBP1 RNA Binding Activity—Transcription factor C/EBPβ plays an important role in liver proliferation after partial hepatectomy (2). It has been recently shown that LAP and LIP isoforms have distinct functions in liver proliferation; whereas LAP regulates timing of cell cycle stages, LIP is required for the induction of cyclins (13). Although C/EBPβ mRNA is increased in liver after PH and this induction contributes to elevation of LAP, the subsequent translation of LIP requires a coordinated cascade of molecular events that direct ribosomes to the third (LIP-specific) AUC codon of the C/EBPβ mRNA. In this paper, we investigated the CUGBP1-dependent mechanism of LIP induction in mouse regenerating livers. Our previous work with rats demonstrated that LIP is increased in rat livers after PH (16). Therefore, we first examined expression of LIP and CUGBP1 after partial hepatectomy in our experimental mice. Since we have previously shown that CUGBP1 activates LIP in mouse livers during acute phase response (APR) (17), this model was used as the control in parallel experiments. Western blotting with antibodies to C/EBPβ is shown in Fig. 1A. In agreement with published observations, expression of both LAP and LIP is increased in mouse liver at 4 and 8 h after PH as well as in livers during early stages of LPS-mediated APR. To elucidate mechanisms by which liver increases translation of LIP, we focused our studies on RNA-binding proteins that might contribute to the induction of LIP during this short time period, 4–8 h after PH. We have previously found that two RNA-binding proteins, CRT and CUGBP1, bind to the 5′ region of C/EBPβ mRNA and might regulate translation of LIP. CRT and CUGBP1 levels were analyzed by Western blot analysis. As can be seen in Fig. 1B, protein levels of CUGBP1 are not altered in regenerating liver or in liver during APR (Fig. 1B). Similar examination of CRT protein levels showed that the levels of this protein declined after PH and during APR (Fig. 1B). Densitometric analysis of CRT levels (relative to β-actin) revealed 2.5–3.5-fold reduction in regenerating livers. Since RNA binding activity of CUGBP1 is also regulated by post-translational modifications (22), we then analyzed the binding activity of CRT and CUGBP1 in mouse liver after PH and APR. For this goal, we used a UV cross-linking assay with the sORF probe (located in the 5′ region of C/EBPβ mRNA; see Ref. 17.
and Fig. 1D), which binds to both CRT and CUGBP1. The sORF probe was incubated with cytoplasmic extracts and cross-linked to proteins by UV irradiation, and CRT and CUGBP1 were immunoprecipitated with specific antibodies. As can be seen in Fig. 1C, binding of CUGBP1 to C/EBPβ mRNA is increased in response to PH and APR at the time points where LIP levels are elevated (Fig. 1A). On the contrary, interactions of CRT with the 5′ region of C/EBPβ mRNA are reduced after PH and during APR consistently with decline of CRT protein levels. These patterns of binding are consistent with the roles played by CRT and CUGBP1 in the regulation of C/EBPβ translation (6, 16). These observations suggested that the proliferating liver increases translation of LIP via an increase of CUGBP1 binding activity and through the elimination of negative effects of CRT. Therefore, we performed further studies to 1) determine whether CRT and CUGBP1 might compete for the regulation of C/EBPβ translation, 2) determine how liver activates CUGBP1 after PH, and 3) elucidate molecular mechanisms of CUGBP1-mediated induction of LIP translation.

**CUGBP1 and CRT Compete for the Regulation of C/EBPβ**—Given the reduction of CRT after PH and our previous data showing that CRT inhibits translation of C/EBPβ (6), we tested whether CRT and CUGBP1 compete for the interaction with the 5′ region of C/EBPβ mRNA. We have previously mapped the CUGBP1 binding site within the 5′ region of C/EBPβ mRNA between the AUG codon of the sORF and the AUG codon for LAP (Fig. 1D). The sORF contains a GC-rich sequence that might form an SL, to which CRT binds (16, 17). Since CUGBP1 binds to the single-stranded sORF RNA, but CRT interacts with the stem-loop structure of the 5′ region of C/EBPβ mRNA, MBP-CUGBP1 and GST-CRT were added to the binding reactions with the sORF probe in 2–4-fold molar excess to the probe. After UV cross-linking, proteins were separated by gel electrophoresis. The membrane was exposed to x-ray film (upper blot) and then probed with antibodies to CRT and to CUGBP1 (Western). Positions of CRT and CUGBP1 are shown.

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C/EBPβ mRNA (Fig. 1D; see Ref. 6), this suggests that CUGBP1 and CRT may compete for the interaction with C/EBPβ mRNA and for the regulation of its translation. To examine this hypothesis, UV cross-linking was performed with bacterially expressed His-CRT and GFP-CUGBP1 under conditions of a 2–4-fold molar excess of these proteins to the sORF probe. Fig. 1E shows the result of a UV cross-linking-Western blotting experiment. When CRT is added to the binding reactions with CUGBP1, it replaces CUGBP1 from the C/EBPβ mRNA. To verify the amounts of CRT and CUGBP1 in the binding reactions, the membrane was probed with antibodies to CRT and to CUGBP1. As can be seen, when both proteins are present in the binding reactions (lanes 2 and 3), CRT blocks the interaction of CUGBP1 with the 5′ region of C/EBPβ. This in vitro result suggested that CRT and CUGBP1 might compete in vivo for the regulation of C/EBPβ mRNA. To test whether CRT and CUGBP1 compete for the regulation of C/EBPβ mRNA, C/EBPβ mRNA (C/EBPβ is linked to the FLAG tag (23)) was co-transfected into HeLa cells with His-CRT and GFP-CUGBP1 at different molar ratios. As can be seen in Fig. 1F, overexpression of CRT inhibits translation of C/EBPβ. However, when GFP-CUGBP1 is expressed to the levels that overcome those of CRT (ratio of plasmids CUGBP1/CRT is 4:1), translation of C/EBPβ is returned to high levels. Since these studies were performed with a co-transfection approach, the contribution of the endogenous CUGBP1 and CRT appears to be minor. Although levels of GFP-CUGBP1 and endogenous 51-kDa CUGBP1 are similar by Western blotting (Fig. 1F), we have obtained 25% efficiency of transfections, suggesting that each transfected cell contains 3–4-fold higher levels of CUGBP1 than cells transfected with His-CRT only. Thus, these data demonstrate that the CRT and CUGBP1 compete for the interaction with the 5′ region of C/EBPβ mRNA and for the regulation of translation of C/EBPβ mRNA. Since the binding of CUGBP1 to the 5′ region of C/EBPβ mRNA is increased in liver after PH, we suggested that the first step in the initiation of LIP translation after PH is the release of the inhibitory activity of CRT.

Translation of LIP Is Increased in Cells Lacking CRT—To further examine the role of competition of CRT and CUGBP1 in the regulation of C/EBPβ mRNA under appropriate biological concentrations and environment, we utilized CRT−/− mouse embryo fibroblasts (22). Western blotting with antibodies to C/EBPβ shows that the expression of both LAP and LIP is increased in cells lacking CRT (Fig. 2A). Densitometric calculations of the levels of LAP and LIP showed that the induction of LIP expression is much higher than the induction of LAP in CRT−/− cells (Fig. 2A, bar graphs). To further examine whether CUGBP1 is involved in the induction of LIP translation in CRT−/− cells, cytoplasm was separated into a polysomal fraction (PS) and a polysome-free fraction (PS-free), and CUGBP1 and CRT were detected by Western blotting with corresponding antibodies. Experimental details for the fractionation of cytoplasm and for examination of polysomal frac-
tions are published in our previous paper (22). Western blotting shows that both CRT and CUGBP1 are observed on polysomes isolated from wild type cells (Fig. 2B). The Western blotting analysis of CUGBP1 on polysomes of CRT−/− cells showed that the lack of CRT correlates with a slight increase of CUGBP1 in polysomal fractions.

To further determine the association of CUGBP1 with polysomes in CRT−/− cells, we utilized a UV cross-linking assay with the sORF probe. Since both CUGBP1 and CRT bind to the sORF probe, we immunoprecipitated these proteins from the binding reactions after UV treatment and loaded on denaturing PAGE. This examination confirmed that CUGBP1 is increased on polysomes of CRT−/− cells (Fig. 2C). These data are consistent with the hypothesis that CRT and CUGBP1 compete for the regulation of C/EBPβ mRNA translation and with the hypothesis that the induction of LIP in the liver after PH might be caused by both the release of CRT-mediated inhibition and activation of CUGBP1. Fig. 2D shows a hypothetical competitive regulation of C/EBPβ by CRT and CUGBP1. Since CRT binds to GC-rich stems, we suggest that CRT stabilizes the SL structure within the 5′ region of C/EBPβ mRNA and partially represses translation. When CUGBP1 is expressed to the levels that overcome those of CRT, it replaces CRT and releases the negative regulation.

**Partial Hepatectomy Causes Hyperphosphorylation of CUGBP1 and Accumulation of the Hyperphosphorylated CUGBP1 on Polysomes**—We further performed a detailed investigation of the mechanisms responsible for activation of CUGBP1 after partial hepatectomy. Since protein levels of CUGBP1 do not correlate with induction of CUGBP1 binding activity, we examined if phosphorylation of CUGBP1 might be involved in its activation after PH. For this goal, we tested the phosphorylation status of CUGBP1 in the cytoplasm of both quiescent livers and livers after PH by using two-dimensional gel electrophoresis. Fig. 3A shows that, in quiescent livers, CUGBP1 exists as four or five isoforms and that hypophosphorylated forms of CUGBP1 (isoforms 3–5) are the major forms. After PH, the amount of the hyperphosphorylated form of CUGBP1 (marked 1, located in the acidic region) is dramatically increased. This isoform of CUGBP1 represents hyperphosphorylated protein, because it is shifted to a more alkaline region by the CIP treatment (Fig. 3A). We have observed that the treatment of CUGBP1 by CIP also leads to a reduced sensitivity of the two-dimensional Western blotting assay. We suggest that this difference might be due to a better interaction of antibodies with phosphorylated CUGBP1.

We have previously shown that CUGBP1 is associated with polysomes that translate C/EBPβ isoforms (16). To determine which isoform of CUGBP1 is involved in the increase of LIP translation, polysomes were isolated from cytoplasmic fractions of livers in 8 h after PH and examined by Western blotting and UV cross-linking assays. These assays demonstrated that hyperphosphorylated isoform of CUGBP1 is abundant in polysomal fractions and is able to bind to C/EBPβ mRNA (Fig. 3B). To determine whether the activation of CUGBP1 is due to phosphorylation, we treated the polysomal fraction with CIP and examined CUGBP1 isoforms by two-dimensional gel electrophoresis and CUGBP1 binding activity by a UV cross-linking assay. Fig. 3C shows that CIP treatment leads to a shift of CUGBP1 into the more alkaline region of the two-dimensional gel. To confirm that dephosphorylation reduces binding activity of CUGBP1, we treated polysomal proteins (isolated at 8 h after PH) with CIP and examined CUGBP1 activity by UV cross-linking assay. The UV cross-linking assay showed that dephosphorylation of CUGBP1 by CIP treatment reduces the CUGBP1 binding activity (Fig. 3C, bottom). This result demonstrates that CUGBP1 is activated after PH by phosphorylation and that the “active” CUGBP1 is located on polysomes. The induction of a hyperphosphorylated form of CUGBP1 in the cytoplasm of regenerating liver suggests that PH activates a kinase that phosphorylates CUGBP1 or inhibits a phosphatase that dephosphorylates CUGBP1. To determine which pathway...
is activated by PH, we incubated bacterially expressed, recombinant CUGBP1 with cytoplasm from livers after 8 h after PH in the presence of ATP and examined its activity after the incubation. As can be seen, the incubation increases binding of CUGBP1 to C/Eβ mRNA, whereas CIP treatment eliminates this effect (Fig. 3D). This result suggests that PH activates a kinase that phosphorylates CUGBP1, leading to the increased interaction of CUGBP1 with the 5' region of the C/Eβ mRNA.

The Association of CUGBP1 with Polysomes Does Not Require Overexpression of RNA Targets—Since CUGBP1 regulates translation of mRNAs on polysomes (17, 22), we examined mechanisms which regulate the association of CUGBP1 with polysomes. Because CUGBP1 binds directly to mRNAs, one would assume that the most likely pathway for the association of CUGBP1 with polysomes is the interaction of this protein with mRNAs. To test this possibility, GFP-CUGBP1 was overexpressed in cells alone or in combination with C/Eβ mRNA, which contains the CUGBP1 binding site, sORF. We have previously shown that another RNA-binding protein, CRT, is associated with polysomes mainly through the interaction with co-transfected mRNAs to which it binds directly (22). Since CRT also interacts with C/Eβ mRNA (6) (see Fig. 1D), we used this protein as the control for the specificity of association of CUGBP1 with polysomes in the presence of C/Eβ mRNA. We have previously found that, although CRT is predominately located in endoplasmic reticulum, a small portion of CRT is also associated with polysomes (22). As can be seen in Fig. 4A, co-transfections of His-CRT with C/Eβ mRNA dramatically increase the amounts of CRT associated with polysomes. On the contrary, the transfection of GFP-CUGBP1 alone is sufficient for the efficient association of CUGBP1 with polysomes. Co-transfection of GFP-CUGBP1 together with C/Eβ mRNA only slightly increases the amounts of CUGBP1 on polysomes. This result shows that the association of CUGBP1 with polysomes does not require overexpression of C/Eβ mRNA, suggesting the existence of other pathways for this association. Therefore, we next examined the hypothesis that association of CUGBP1 with polysomes is mediated through interactions of CUGBP1 with other proteins.
CUGBP1 Interacts with the α and β Subunits of Eukaryotic Initiation Factor eIF2—To identify proteins that interact with CUGBP1, MBP-CUGBP1 was linked to amylose beads and incubated with cytoplasmic proteins from HeLa cells. After extensive wash and elution, MBP-CUGBP1 binding proteins were separated by gel electrophoresis. A typical picture of the Coomassie Blue staining is shown in Fig. 4B. We reproducibly observed five proteins from HeLa cells that interact with MBP-CUGBP1. A protein with a molecular mass of 60 kDa was eluted from the membrane and sequenced. Peptide analysis showed that this protein is β-tubulin. We are currently investigating a possible biological role of the interaction of CUGBP1 with β-tubulin. Attempts to determine sequences of other MBP-CUGBP1-binding proteins were unsuccessful; therefore, we used alternative approaches for their identification. One of the proteins interacting with MBP-CUGBP1 was migrating in the position of 51 kDa, which corresponds to the size of CUGBP1. Since our previous studies suggested that CUGBP1 might bind to RNA as a homodimer (22), we examined whether the 51-kDa protein might be CUGBP1. To test this suggestion, we incubated purified MBP-CUGBP1 with nuclear extracts from HeLa cells that contain high levels of CUGBP1. Pull-down samples were analyzed by Western blotting with CUGBP1 antibodies. As shown on Fig. 4C, MBP-CUGBP1 interacts with endogenous 51-kDa CUGBP1.

Two other MBP-CUGBP1-interacting proteins have molecular masses of 35 and 52 kDa. Since cytoplasmic CUGBP1 regulates translation of mRNAs, we suggested that these proteins might be eukaryotic translation factors. It has been shown that eukaryotic initiation translation factor subunits eIF2α, eIF2β, eIF2γ, and eIF4E play a critical role in initiation of protein synthesis (24, 25). Several previous observations suggested that eIF2α might be involved in the control of LIP translation. First, experiments from Dr. Rosen’s laboratory showed a correlation between elevation of LIP expression and induction of eIF2α in breast tumors, suggesting that eIF2α is involved in the regulation of C/EBPβ translation (26). Moreover, Calkhoven et al. (14, 15) have shown that overexpression of eIF2α in 3T3-L1 cells increases translation of LIP. Therefore, we examined if CUGBP1 can interact with eukaryotic initiation factors eIF2α, eIF2β, eIF2γ, eIF3α, and eIF4E. A fusion MBP-CUGBP1 protein attached to amylose beads was incubated with cytoplasmic proteins isolated from HeLa and from COS7 cells. Bound proteins were eluted from the column with high salt and separated by the denaturing gel electrophoresis. Western blotting showed that eIF2α interacts with CUGBP1, whereas eIF4E does not show detectable interactions (Fig. 4D).

The interaction of CUGBP1 with eIF2α does not appear to occur through the binding of the individual proteins to RNAs, since eIF4E (which is also an RNA-binding protein) is not observed in the complexes with CUGBP1. eIF2α displays its translational functions as a heterodimer with eIF2β and eIF2γ subunits (25). Given the interaction of CUGBP1 with eIF2α, we examined a composition of eIF2α ternary complex that binds to CUGBP1. CUGBP1 pull-down samples were examined by Western blotting with antibodies to eIF2β and eIF2γ. Fig. 4D shows that eIF2β also binds to CUGBP1. Under the sensitivity of our assay, the interaction of eIF2γ with CUGBP1 was not detectable. We also could not detect the interaction of CUGBP1 with another initiation factor, eIF3α, using an MBP pull-down assay. Since the molecular mass of eIF2α is 36 kDa and that of eIF2β is 52 kDa, we suggest that these proteins are observed in pull-down samples presented on Fig. 4B. Thus, these studies showed that CUGBP1 specifically interacts with the α and β subunits of the eIF2.

We next examined whether CUGBP1 interacts with eIF2α in vivo under physiological concentrations and environment. eIF2α and eIF4E were immunoprecipitated from HeLa cytoplasm, and CUGBP1 was examined in immunoprecipitations by Western blotting with monoclonal antibodies. Fig. 4E shows that CUGBP1 is observed in immunoprecipitations with eIF2α, whereas immunoprecipitates of eIF4E do not contain detectable amounts of CUGBP1. We next mapped the region of CUGBP1 that interacts with eIF2α. An MBP pull-down assay with different portions of CUGBP1 shows that RBD1 interacts with eIF2α, whereas RBD2 and RBD3 do not interact with eIF2α (Fig. 4F). A reprobe of the filter with antibodies to eIF4E showed that the interaction of RBD1 with eIF2α is specific.

CUGBP1-eIF2α/β Complexes Are Associated with RNA—Since CUGBP1, eIF2α, and eIF2β might be associated with each other indirectly, through independent bindings to the same mRNAs, we next examined whether RNA molecules contribute to the interactions of these proteins. For this goal, we applied HPLC-based size exclusion chromatography technique using an SEC-400 column (Bio-Rad). Western analysis of CUGBP1 in gel filtration fractions showed that, despite the fact that CUGBP1 is a 51-kDa protein, the major fraction of CUGBP1 is detected in fractions containing high molecular weight RNA-protein or protein-protein complexes (Fig. 5). This result is consistent with previously published observations (27). Western blotting for eIF2α and eIF2β shows that two regions of gel filtration fractions contain these proteins; one region co-localizes with the position of CUGBP1, and the second region corresponds to the size of free eIF2α and eIF2β. Note that we have examined CUGBP1, eIF2α, and eIF2β that are not associated with polysomes or with 40 S ribosomal subunit, since those are located in the void volume excluded from this analysis. Co-immunoprecipitation experiments showed that CUGBP1 and eIF2α and eIF2β form complexes, the size of which ranged from 400 to 600 kDa. Since the size of the CUGBP1-eIF2α/β complex is much bigger than one would predict from the individual sizes of these proteins, we examined whether RNA transcripts might contribute to the interaction of CUGBP1 with eIF2α. Cytoplasmic proteins from HeLa cells were treated with RNase A and separated on a size exclusion SEC-400 column. Examination of size exclusion fractions of HeLa cytoplasm treated with RNase A by Western blotting with antibodies to CUGBP1, eIF2α, and eIF2β showed that these proteins are shifted to lower molecular weight fractions by RNase A treatment. However, these proteins still exist as a complex with a approximate molecular mass of 200–300 kDa. Thus, the HPLC-based analysis of CUGBP1-eIF2α/β complexes demonstrates that CUGBP1 interacts with eIF2α and eIF2β and that the CUGBP1-eIF2α/β complex is associated with RNAs in the cytoplasm of HeLa cells.

Dephosphorylation of CUGBP1 Reduces Its Interaction with eIF2α and with eIF2β—Analysis of CUGBP1 in mouse liver and in cultured cells revealed that the hyperphosphorylated form of CUGBP1 is associated with polysomes (Figs. 2 and 3). In addition, Co-immunoprecipitation studies also suggested that the hyperphosphorylated form of CUGBP1 interacts with eIF2α (Fig. 4). Therefore, we tested the hypothesis that phosphorylation of CUGBP1 might regulate interactions with eIF2α and perhaps the association of CUGBP1 with mRNAs. To test this hypothesis, we utilized a liver regeneration model in which partial hepatectomy causes hyperphosphorylation of CUGBP1 (Fig. 3). Cytoplasmic extracts from quiescent livers and from livers 8 h after PH were fractionated by size exclusion chromatography. Examination of CUGBP1, eIF2α, and eIF2β in gel filtration fractions is shown in Fig. 6. In quiescent livers, the major portion of CUGBP1 is observed in fractions with molecular mass ranging from 40 to 200 kDa, whereas eIF2α is
located throughout all fractions, with maximum levels in the high molecular mass region of gel filtration. At 8 h after PH, a portion of CUGBP1 is shifted to the high molecular mass region, where it co-localizes with both eIF2α and eIF2β and forms complexes with eIF2α, since eIF2α is observed in CUGBP1 immunoprecipitations from these fractions. The formation of CUGBP1-eIF2α/β complexes 8 h after PH is dependent on phosphorylation of CUGBP1, because CUGBP1-eIF2α/β complexes are not detectable after treatment of cytoplasmic extracts with CIP (Fig. 6, bottom image). Thus, these studies suggest that the hyperphosphorylated form of CUGBP1 interacts with eIF2α and eIF2β in mouse liver after PH and that the dephosphorylation of cytoplasmic extracts leads to a disruption of the CUGBP1-eIF2α/β complexes.

**DISCUSSION**

A member of the C/EBP family, C/EBPβ, is an important regulator of cell growth, differentiation, and immune response (1–4). Both full-length C/EBPβ protein and the truncated isoform LIP are induced in regenerating livers (16, 28). A recent paper demonstrated that LIP has an independent function in proliferating livers, which is the activation of S-phase-specific genes such as cyclins E and A and proliferating cell nuclear antigen (13). Molecular mechanisms by which liver increases translation of LIP are not well understood. A number of recent observations showed that expression of many cell cycle proteins is regulated by RNA-binding proteins at the level of translation and mRNA stability (5, 29–32). Expression of C/EBP proteins and their isoforms is also controlled by RNA-binding proteins. A misregulation of this control in cells might cause dramatic alterations in biological processes. Perrotti et al. (5) have shown that the BCR-ABL oncogene causes the development of myeloid leukemia through activation of the poly(rC)-binding protein, which interacts with C/EBPα mRNA and blocks translation of C/EBPα. Searching for RNA-binding proteins that regulate translation of C/EBPα mRNA, we have found that CRT also inhibits translation of the C/EBPα mRNA (6). Consistent with
this finding, recent work from Dr. Pabst's group demonstrated that a leukemic fusion gene, AML-MDS1-EVI1, causes acute myeloid leukemias by activating C/EBP and with the subsequent suppression of C/EBPα (32). In addition to the regulation of C/EBPα, CRT also regulates translation of p21 and C/EBPβ and might control cell growth through these interactions (6, 22).

In this paper, we examined the pathway by which CUGBP1 increases translation of LIP in mouse liver after PH. We found that the binding activities of CRT and CUGBP1 are irreversibly altered in mouse livers after partial hepatectomy. Our experiments in cultured cells revealed that CRT and CUGBP1 compete for the interaction with eIF2α mRNA and for the regulation of its translation (Figs. 1 and 2), suggesting that the activated CUGBP1 replaces CRT from the 5′ region of C/EBPβ mRNA after PH. The competitive regulation of mRNAs by CUGBP1 and CRT seems to have a more broad significance and is not limited to the regulation of C/EBPβ mRNA. We have previously found that the 5′ region of p21 mRNA also contains overlapping binding sites for CUGBP1 (single-stranded GCN repeats) and for CRT (GC-rich SL structure). Similar to the regulation of C/EBPβ mRNA, the translation of p21 mRNA is also under the control of CRT and CUGBP1. We found that this competitive regulation plays a role in the induction of p21 during replicative senescence (22). Interestingly, the pathways for the regulation of CRT and CUGBP1 seem to be similar in senescent fibroblasts and in regenerating livers. Whereas the reduction of CRT is associated with a decline of the protein levels, CUGBP1 is activated by phosphorylation in both senescent fibroblasts and regenerating livers (see Fig. 1 and Ref. 22).

Although previous studies implicated eIF2α in the translational regulation of LIP (15, 26), the molecular mechanism by which eIF2α selectively directs the initiation of translation from the LIP-specific AUG codon has not been elucidated. Our failure to detect eIF2α from the LIP-specific AUG codon suggests that the 3′ LIP-specific AUG codon has not been elucidated. Our findings from the binding of CUGBP1 to eIF2, which then facilitates the release of eIF2γ, the binding of CUGBP1 to free eIF2α and eIF2β (or eIF2αβ) that are part of the pool for the synthesis of eIF2, or the artificial release of the eIF2γ from a CUGBP1-eIF2 complex that breaks down slightly during our isolation methodologies. Our experimental design for the HPLC-based gel filtration analysis was focused on the interactions of CUGBP1 with eIF2, which is free of ribosomes. It is possible that the eIF2γ might be associated with CUGBP1-eIF2αβ when the CUGBP1-eIF2αβ complex reaches polysomes. In addition to C/EBPβ, CUGBP1 also binds to and increases translation of several skeletal muscle mRNAs, such as p21 and MEF2A (34, 35). Although the mechanisms of this increase might differ from one proposed for the translational induction of LIP, the interaction of CUGBP1 with subunits of eIF2 might also be involved in these effects and thereby might control biological processes such as myogenesis.

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