Calmodulin Controls Liver Proliferation via Interactions with C/EBPβ-LAP and C/EBPβ-LIP*

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A truncated isoform of C/EBPβ, C/EBPβ-LIP, is required for liver proliferation. This isoform is expressed at high levels in proliferating liver and in liver tumors. However, high levels of C/EBPβ-LIP are also observed in non-proliferating livers during acute phase response (APR). In this paper we present mechanisms by which liver regulates activities of C/EBPβ-LIP. We found that calmodulin (CaM) inhibits the ability of C/EBPβ-LIP to promote liver proliferation during APR through direct interactions. This activity of CaM is under negative control of Ca2+, which is reduced in nuclei of livers with APR, whereas it is increased in nuclei of proliferating livers. A mutant CaM, which does not interact with C/EBPβ-LIP, also fails to inhibit the growth promotion activity of C/EBPβ-LIP. Down-regulation of CaM in livers of LPS-treated mice causes liver proliferation via activation of C/EBPβ-LIP. Overexpression of C/EBPβ-LIP above levels of CaM also initiates liver proliferation in LPS-treated mice. In addition, CaM regulates transcriptional activity of another isoform of C/EBPβ, C/EBPβ-LAP, and might control liver biology through the regulation of both isoforms of C/EBPβ.

In searching for molecular mechanisms by which C/EBPβ-LIP promotes cell proliferation, we found that C/EBPβ-LIP releases E2F-Rb-dependent repression of cell cycle genes by a disruption of E2F1-Rb complexes and by a direct interaction with E2F-dependent promoters. CaM inhibits these growth promotion activities of C/EBPβ-LIP and, therefore, supports liver quiescence. Thus, our findings discover a new pathway of the regulation of liver proliferation that involves calcium-CaM signaling.

Liver proliferation is controlled by a complex cooperation of several signal transduction pathways (1–4). A member of C/EBPβ family, C/EBPβ, is one of the key proteins that regulates liver proliferation (5, 6). Although C/EBPβ is an intronless gene, a single C/EBPβ mRNA produces three isoforms: full-length protein, liver activator protein (LAP), and liver inhibitor protein (LIP) (7, 8). C/EBPβ-LIP lacks activation domains and works as a dominant negative molecule by neutralizing activities of full-length C/EBP proteins (7, 9). C/EBPβ-LIP also interacts with other transcription factors and displays its activity through these interactions (10, 11). Partial hepatectomy increases expression of C/EBPβ mRNA at early times, resulting in elevation of all three isoforms of C/EBPβ (5, 12, 13). The alternative translation of C/EBPβ-LIP isoform requires activation of signal transduction pathways which increase initiation of translation from the third AUG codon. One of these pathways is controlled by RNA-binding protein CUGBP1. It has been shown that CUGBP1 directly binds the 5′ region of C/EBPβ mRNA and increases translation of C/EBPβ-LIP (12, 13). We have recently identified mechanisms by which CUGBP1 increases translation of C/EBPβ-LIP. These mechanisms include activation of CUGBP1 by cyclin D3-cdk4/6, which enhances interactions of CUGBP1 with translation initiation complex eIF2 and leads to recruitments of ribosomes to translate C/EBPβ mRNA (14, 15). The CUGBP1-eIF2-dependent activation of C/EBPβ-LIP has been observed in three biological situations; that is, in livers proliferating after partial hepatectomy (PH), in the liver during acute phase response (APR), and in livers of old mice (9, 13–15). Further studies have shown that elevation of C/EBPβ-LIP leads to the acceleration of proliferation of certain cultured cells (16). The elevation of C/EBPβ-LIP also leads to aggressive forms of breast cancer (17). Luedde et al. (18) have shown that C/EBPβ-LIP accelerates liver proliferation after PH by activation of PCNA and cyclin A.

Calmodulin (CaM) is a calcium-binding protein that is a common sensor for intracellular calcium signaling (19). CaM has no enzymatic activity and functions mainly as the translator of calcium signaling. There are several pathways by which CaM translates calcium signaling; that is, CaM-dependent phosphatases, CaM-dependent kinases, the transcription corepressors Cabin1, and histone deacetylase (19–21). In addition to these pathways, CaM directly interacts with transcription factors (calmodulin binding transcription activators) and might control growth and differentiation of several tissues (22). Several recent reports have suggested that CaM might regulate cell proliferation via different mechanisms. It has been shown that insulin-mediated stimulation of fibroblasts proliferation involves activation of calcium-CaM-CaM kinase II pathway (23). Choi et al. (24) have found that CaM regulates proliferation of vascular smooth muscle cells via interactions with cyclin E (26). Calmodulin also interacts with cyclin-dependent kinase inhibitor p21 and controls nuclear localization of p21 (27, 28).

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*The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; LAP, liver activator protein; LIP, liver inhibitor protein; APR, acute phase response; CaM, calmodulin; CUGBP1, CUG triplet repeat-binding protein; LPS, lipopolysaccharide; EMSA, electromophoretic mobility shift assay; PH, partial hepatectomy; IP, immunoprecipitation; siRNA, small interfering RNA; GST, glutathione S-transferase; DHFR, dihydrofolate reductase; BrdUrd, bromodeoxyuridine; Abs, antibodies; WT, wild type.

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C/EBPβ-LIP is increased in non-proliferating livers during APR (9, 13) and in livers of old mice, which is characterized by reduced proliferative capacities (14, 29, 30). Given the ability of C/EBPβ-LIP to accelerate liver proliferation after PH (18), we suggested that livers with APR have developed a mechanism that blocked growth promotion activities of C/EBPβ-LIP. In this paper, we have examined this hypothesis using LPS-mediated activation of APR in mouse livers. We found that C/EBPβ-LIP promotes proliferation via interaction with and disruption of Rb-E2F complexes and that CaM blocks these growth promotion activities of C/EBPβ-LIP in livers of LPS-treated mice. The down-regulation of CaM in LPS-treated mice initiates liver proliferation by a release of growth promotion activities of C/EBPβ-LIP.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—** Antibodies against C/EBPα (14AA), C/EBPβ (C-19), Rb (C-15), E2F1 (KH95), and E2F4 (C-20) were purchased from Santa Cruz Biotechnology. Antibodies to calmodulin and β-actin were from Millipore and Sigma, respectively. Antibodies to total Rb, to ph-Ser-612-Rb, and to ph-Ser-811-Rb were from Millipore. True-Blot secondary antibodies and IP beads were from Ebioscience. siRNAs to C/EBPβ and calmodulin were from Dharmacon. LPS and BrdUrd were from Sigma. The BrdUrd uptake assay kit and Fura-2 were from Invitrogen.

**Generation of p3XFLAG-C/EBPβ-LIP Δ(264–296) Mutant—** Mutations were constructed using the QuikChange™ XL site-directed mutagenesis kit from Stratagene. A plasmid p3XFLAG-C/EBPβ-LIP was used as a template, and PCR amplification was performed in the presence of a forward primer, GCGGAGAACGAGCCGTCTAGAGATCCGG, and a reverse primer, CCGGGATCCTCTAGACCGCTCGTCTCCGC. HEK293 cells were co-transfected with p3XFLAG-C/EBPβ-LIP Δ(264–296) and pAd-Track-CaM. The presence of C/EBPβ-LIP Δ(264–296) in CaM IP was examined by Western blotting using FLAG-horseradish peroxidase from Sigma.

**Animals and Experiments with LPS, C/EBPβ, and CaM siRNA—** All research protocols for animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol #AN1349). 2–4-month-old mice were used for experiments described in this paper. The FLAG-C/EBPβ-LIP and calmodulin siRNA with or without C/EBPβ siRNA were delivered into mice by tail vein injection using the “in vivo-jetPEI transfection reagent” from PolyPlus Transfection. LPS was injected intraperitoneally on the next day. Because the proliferation of hepatocytes might be initiated by C/EBPβ-LIP in non-synchronized manner, BrdUrd was injected every day to provide higher BrdUrd incorporation. Physiological saline (0.9% NaCl) was used as a control. Liver samples were collected and kept at −80 °C. Data in the paper represent a summary of three experiments with 3–4 animals at each time point after LPS injection.

**Cell Culture and Transient Transfection—** HEK293 and Hep3B2 cell lines (from ATCC) were cultured in monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone) with 100 units/ml penicillin-streptomycin (Invitrogen). The cells were grown at 37 °C in a humidified incubator with 5% CO₂. Cells were transfected with pAdTrack-C/EBPβ-LIP, pAdTrack-CaM, or siRNA to CaM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 16 h after transfections, the cells were washed with phosphate-buffered saline and collected in phosphate-buffered saline. Protein extracts were isolated as described below.

**Isolation of Proteins and Western Blotting Analysis—** Cytoplasmic and nuclear extracts were isolated as described in our previous papers (30, 31). Briefly, cell pellets were resuspended in buffer A (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, and inhibitors of phosphatases) and homogenized by shearing the suspension through an insulin syringe. After centrifugation, the supernatant (cytoplasm) was frozen and kept in the −80 °C freezer. The procedure of isolation of cytoplasmic and nuclear extracts from liver was similar to that described above except that liver was homogenized in Buffer A by using a homogenizer. 50–100 μg of proteins were fractionated by SDS-PAGE using 4–20% gels, electrophoretically transferred to nitrocellulose membrane, and then probed with the indicated antibodies. The signals were detected by ECL Western blotting detection reagents (Amersham Biosciences). Protein loading was verified by the re-probe of the membranes with antibodies to β-actin and by Coomassie Stain.

**Two-dimensional Examination of the Proteins That Differentially Interact with C/EBPβ-LIP—** GST-C/EBPβ-LIP was incubated with 1000 μg of nuclear proteins isolated from liver at 8 h after PH and with proteins isolated from livers at 8 h after LPS administration. After intensive wash with phosphate-buffered saline, the proteins were eluted and separated by two-dimensional gel electrophoresis on strips pH 3–10 using Protean II (Bio-Rad) and after electrophoresis in 4–20% polyacrylamide gel. The gels were stained with silver, and the spots with differential intensities were cut out and sequenced. The differential interaction of Rb and CaM with C/EBPβ-LIP was further confirmed by a GST pulldown assay and co-IP approaches.

**Co-IP Approach—** HEK293 or Hep3B2 cells were transfected with pAdTrack-LIP or pAdTrack-CaM, respectively. 500 μg of total cell lysates were used for immunoprecipitation. The co-IP studies were performed as described in our publications (15, 30, 31). In the experiments with increasing concentrations of EDTA or/and Ca²⁺, cell lysates with CaM transfections were preincubated with 2 and 4 mM EDTA or with 2 and 4 mM CaCl₂ on ice for 1 h. Then, protein extracts from HEK293 cells transfected with C/EBPβ-LIP were added to these mixtures, and C/EBPβ-LIP was precipitated with antibodies to C/EBPβ. The rabbit IgG Trueblot beads were added to the mixture and incubated at 4 °C for overnight. The immunoprecipitates were resolved on a 4–20% SDS-PAGE followed by immunoblotting with the indicated antibodies. For the immunoprecipitation of C/EBPβ from liver nuclear extracts, 500 μg of nuclear extracts from mouse livers were incubated with 4 mM EDTA or CaCl₂.
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overnight. The rabbit IgG Trueblot beads and antibodies to C/EBPβ were added and incubated for 2 h. The presence of CaM in C/EBPβ IP was examined by Western blotting. All data in the manuscript represent the results of the 3–4 repeats.

**EMSA Assay**—Conditions for the EMSA (or gel shift) assay were described in our previously papers (30, 31). Briefly, the 32P-labeled DHFR, E2F, and cdc2 oligomers were used as the probes. For examination of E2F-Rb complexes, nuclear extracts from HEK293 cells transfected with C/EBPβ-LIP or C/EBPβ-LIP and CaM were incubated with probes in the binding reactions containing salmon DNA as a competitor. Antibodies to E2F1, Rb, E2F4, and p130 were added before the probe addition. For examination of the direct interactions of C/EBPβ-LIP with E2F consensuses, poly(dI-dC) was used as nonspecific competitor. It has been shown that poly(dI-dC) inhibits E2F binding but does not affect interactions of C/EBP proteins with DNA (30, 31). DNA-protein complexes were separated by non-denaturing 6% polyacrylamide gel electrophoresis in 0.5× Tris borate EDTA buffer. After electrophoresis, the gel was dried and exposed to x-ray film. Each EMSA experiment was repeated 3–4 times with different transfections of the proteins.

**Chromatin-IP Studies**—A chromatin-IP assay was performed as described in our previous papers using a Chip-It kit (30, 31). Briefly, chromatin solutions were prepared from HEK293 cell transfected with FLAG-C/EBPβ-LIP, with empty FLAG vector, with CaM, or mutant calmodulin CaMΔ. E2F1, Rb, or FLAG were immunoprecipitated from the solutions. DNA was isolated and used for PCRs with primers covering E2F sites within the B-myb and DHFR promoter. The sequences of primers for the B-myb promoter were 5′-CCGGACTGACA-CGTGACGC-3′ (forward) and 5′-GTCAGGCTGTCAAGCA GGTC-3′ (reverse). The sequences of DHFR primers were 5′-CTGCAAAATGGGGAGCAAGCAGA-GGTC-3′ (forward) and 5′-CCA TGTCTGGGACGACGC-3′ (reverse).

**BrdUrd Uptake**—BrdUrd was injected every day after LPS treatments. Liver sections were fixed in 10% formalin. BrdUrd staining was performed using a BrdUrd uptake assay kit from Invitrogen according to the manufacturer’s protocol. Examination of BrdUrd uptake was performed using three animals per each time point after LPS injection.

**[Ca2+] Measurement**—Ca2+ concentration was measured by using calcium-sensitive indicator Fura-2 (32, 33). Levels of Fura-2 fluorescence intensity of nuclear extracts containing 1 μM Fura-2 were measured at 340-nm and 380-nm excitations (excitation scan) and 510-nm emission wavelengths using an F-4500 fluorescence spectrophotometer.

**Colony Formation Assay**—The ability of C/EBPβ-LIP to promote cell proliferation was examined using colony formation assay. This approach has been previously developed in our laboratory, and it is one of the best procedures for the analyses of cell proliferation/inhibition (31). This approach allows verification of the expression of transfected proteins in the experimental plates in the end of experiments. The HEK293 or Hep3822 cells were plated at low density when each colony represents a single cell in the beginning of experiments. These cells were transfected with AdTrack-C/EBPβ-LIP plasmid, which expresses C/EBPβ-LIP and green fluorescent protein (GFP) from different promoters (see Fig. 6A). Therefore, each green cell also expresses C/EBPβ-LIP. Control cells were transfected with an empty AdTrack plasmid. The number of cells in each colony was examined at days 2, 3, and 4 after transfections. The colonies with two and more cells were considered as proliferating cells, whereas single cells are considered as growth-arrested cells. The rate of cell proliferation was calculated by counting the percentage of proliferating cells transfected with empty vector and with C/EBPβ-LIP plasmid. 150–200 cells were used for these calculations. After calculations, protein extracts were isolated from the experimental plates and used for Western blotting analyses as is shown in Fig. 6D. Data in the paper present results of three-four independent experiments.

**RESULTS**

**Identification of Proteins That Differentially Interact with C/EBPβ-LIP in Nuclear Extracts from Proliferating Livers and from Livers with APR**—Previous studies have shown that C/EBPβ-LIP is increased in livers proliferating after PH and in livers during APR (9, 13, 15). We have first examined if the elevation of C/EBPβ-LIP is comparable in these settings. APR was initiated by injections of LPS as described (13). Western blotting confirmed previous findings and revealed a significant increase of LIP in these two biological situations (Fig. 1A). Calculations of the levels of C/EBPβ-LIP revealed that expression of C/EBPβ-LIP, as a ratio to β-actin, is 6–8-fold increased in livers after PH and in livers with APR (Fig. 1A, bar graphs).

Because the elevation of C/EBPβ-LIP after PH promotes liver proliferation but identical levels of C/EBPβ-LIP after LPS injection do not initiate liver proliferation, we suggested that the biological activities of C/EBPβ-LIP are regulated in these settings by differentially interacting proteins. To examine this hypothesis, we have isolated and sequenced proteins that differentially bind to C/EBPβ-LIP in livers proliferating after PH and in livers with APR. For this goal GST-C/EBPβ-LIP was incubated with protein extracts of proliferating livers (8 h after PH) and with protein extracts isolated from livers with APR (8 h after LPS injection). We have observed several spots with different intensities on two-dimensional gels (Fig. 1B). The spots with significant differences in intensity were sequenced in the Protein Chemistry Core Laboratory at Baylor College of Medicine. We found that Rb, Grp78, and Hsp70 strongly interact with GST-C/EBPβ-LIP in nuclear extracts from proliferating livers, whereas mouse ATP synthase, cytochrome b6, major mouse urinary protein, and CaM strongly interact with GST-C/EBPβ-LIP in extracts from livers with APR (Fig. 1C). We have further focused our studies on the interactions of C/EBPβ-LIP with Rb and CaM as Rb has been shown to be a mediator of C/EBPβ-LIP activities (34–36) and because CaM regulates activities of transcription factors (22).

The Reduction of Ca2+ in Nuclei of LPS-treated Mice Increases the Interaction of CaM with C/EBPβ-LIP and with C/EBPβ-LAP—Because the interaction of CaM with GST-C/EBPβ-LIP is increased without alterations in protein levels of CaM and Rb (see Figs. 2, B and C), we have looked for the mediators that might regulate this interaction. It has been shown that the interaction of CaM with proteins depends on the concentration of Ca2+ (19). Therefore, we examined the interactions of C/EBPβ-LIP and CaM under different concen-
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trations of Ca\^{2+}. C/EBP\(\beta\)-LIP and CaM were overexpressed in cultured cells and incubated with each other in buffers containing increasing concentrations of Ca\(^{2+}\) and EDTA (2 and 4 mM each). Fig. 2A shows that the elimination of Ca\(^{2+}\) by EDTA significantly increases interactions of CaM with C/EBP\(\beta\)-LIP, whereas the increase of concentration of Ca\(^{2+}\) significantly reduces this interaction. Calculations of a ratio of CaM to C/EBP\(\beta\)-LIP within C/EBP\(\beta\) IPs show a 2–3-fold increase of the interaction by EDTA and around a 4-fold reduction of interaction by high concentrations of Ca\(^{2+}\). We next determined concentrations of Ca\(^{2+}\) in nuclear extracts isolated from control livers and from livers at 4 and 8 h after injection of LPS and found that the concentration of Ca\(^{2+}\) is reduced in nuclei of LPS-treated mice (Fig. 2B).

We next determined if endogenous CaM and C/EBP\(\beta\)-LIP interact with each other in the liver. The studies of endogenous C/EBP\(\beta\)-LIP in the liver are complicated by the fact that both C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LIP are expressed from the single mRNA and by the fact that there are no available antibodies to C/EBP\(\beta\)-LIP to distinguish this isoform from C/EBP\(\beta\)-LAP. Therefore, our experiments with antibodies to C/EBP\(\beta\) (C19, Santa Cruz) show interactions of CaM with both isoforms of C/EBP\(\beta\). We first immunoprecipitated C/EBP\(\beta\) from nuclear extracts of livers harvested at 0, 4, and 8 h after LPS injection, and CaM was examined in these IPs. We found that the interactions of the endogenous C/EBP\(\beta\) with CaM are increased in LPS-treated mice at 4 and 8 h. To determine whether Ca\(^{2+}\) plays a causal role in the interactions of C/EBP\(\beta\)-LIP and CaM, we have preincubated nuclear extracts with Ca\(^{2+}\) and found that the restoration of Ca\(^{2+}\) concentration in nuclear extracts inhibits interactions of CaM with C/EBP\(\beta\)-LIP (Fig. 2B). Immunoprecipitation of CaM and Western blotting with Abs to C/EBP\(\beta\) confirmed that the association of CaM with C/EBP\(\beta\)-LIP is increased in LPS-treated mice (Fig. 2C). These studies have shown that both C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LAP isoform bind to CaM.

Our GST pulldown experiments demonstrated that the interactions of C/EBP\(\beta\)-LIP with Rb are reduced in nuclear extracts from LPS-treated mice compared with interactions in regenerating livers (Fig. 1). Therefore, we next examined interactions of endogenous C/EBP\(\beta\)-LIP with Rb are altered in livers of LPS-treated mice. For this goal, C/EBP\(\beta\) was immunoprecipitated, and Rb was examined in these IPs. Fig. 2D shows that the association of C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LAP with Rb is reduced at 4 and 8 h after LPS treatment. Taken together, examination of early steps of APR showed that the interactions of C/EBP\(\beta\)-LIP with Rb are reduced, whereas the interactions of C/EBP\(\beta\)-LIP with CaM are increased in the liver.

The Increase of Ca\(^{2+}\) in Nuclei of Proliferating Livers Leads to the Inhibition of Interactions of C/EBP\(\beta\)-LIP with CaM—We next determined if endogenous C/EBP\(\beta\)-LIP interacts with CaM in livers proliferating after partial hepatectomy, PH. Examination of Ca\(^{2+}\) in nuclei of regenerating livers showed an increase of Ca\(^{2+}\) at 4–48 h after PH (Fig. 2E, bar graphs). Levels of CaM after PH were not changed significantly in nuclei of livers at 4–48 h after PH, whereas levels of C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LAP were increased (Fig. 2E). We next precipitated C/EBP\(\beta\)-LIP from nuclear extracts of regenerating livers and examined CaM and C/EBP\(\beta\)-LIP in these IPs. We found that CaM is not detectable in C/EBP\(\beta\) IPs from regenerating livers (Fig. 2F, upper image). The elevation of Ca\(^{2+}\) seems to be a major cause of the lack of interactions as the elimination of Ca\(^{2+}\) from nuclear extracts by EDTA increases interactions of C/EBP\(\beta\)-LIP and CaM at 8–48 h after PH. Thus, these studies demonstrated that the increase of Ca\(^{2+}\) in nuclei of regenerating livers prevents the interaction of CaM with C/EBP\(\beta\)-LIP.
Identification of Regions of CaM and C/EBP\(\beta\)-LIP, Which Are Required for the Interactions—For the investigations of the effects of CaM on activities of C/EBP\(\beta\)-LIP, we have generated a mutant CaM\(\Delta\) that does not interact with C/EBP\(\beta\)-LIP. CaM consists of two domains linked by a spacer, which is critical for the “active” conformation of CaM (19). Therefore, we have generated the mutant CaM (linked to V5 tag) with the deletion of six amino acids within the spacer region (Fig. 3A). Co-IP studies have shown that the mutant CaM\(\Delta\) does not bind to C/EBP\(\beta\)-LIP; whereas WT CaM interacts with C/EBP\(\beta\)-LIP (Fig. 3B).

To determine regions of C/EBP\(\beta\)-LIP that interact with CaM, we generated several FLAG-linked C/EBP\(\beta\)-LIP-truncated molecules and examined these mutants for the interactions with CaM. However, the small deletions were not efficient to block these interactions. We found that deletion of a long region from the C terminus completely blocks interactions of C/EBP\(\beta\)-LIP with CaM (Fig. 3, C and D). The deleted region is 32 amino acids long and includes three leucine residues from the zipper region of C/EBP\(\beta\)-LIP. The identification of the leucine zipper of C/EBP\(\beta\)-LIP as the CaM-interacting region raised a possibility that CaM might also affect transcriptional activity of the C/EBP\(\beta\)-LIP isoform, which contains the zipper region and which also interacts with CaM (see Fig. 2). Therefore, we have examined if CaM regulates transcriptional activity of C/EBP\(\beta\)-LIP. For this goal, C/EBP\(\beta\)-LIP was co-transfected with C/EBP-dependent C3-luc reporter plasmid and with increasing amounts of a plasmid coding for CaM. Fig. 3E shows that CaM inhibits transcriptional activity of C/EBP\(\beta\)-LIP. This inhibition is specific as CaM does not affect the translational activity of SMAD2 and because CaM\(\Delta\) mutant does not inhibit C/EBP\(\beta\)-LIP. These studies suggested that CaM might regulate activities of both isoforms of C/EBP\(\beta\), LAP and LIP. Therefore, we next examined if interactions of CaM with C/EBP\(\beta\)-LAP and C/EBP\(\beta\)-LIP control activities of these proteins in the liver.

Down-regulation of CaM in Livers of LPS-treated Mice Causes Liver Proliferation via Activation of C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LAP—During liver regeneration after PH, C/EBP\(\beta\)-LIP, and C/EBP\(\beta\)-LAP are elevated (Fig. 2E) and are required for proper liver proliferation (5, 6). However, a similar elevation of C/EBP\(\beta\) isoforms after LPS treatments is not sufficient to initiate liver proliferation (see Fig. 1 and Ref. 13). Because CaM interacts with C/EBP\(\beta\)-LIP and C/EBP\(\beta\)-LAP in LPS-treated mice, we suggested that the lack of liver proliferation in LPS-treated mice might be due to the increased interactions of C/EBP\(\beta\) with CaM. To test this suggestion, we have inhibited CaM by siRNA in the liver of mice treated with LPS and examined liver proliferation after PH. C/EBP\(\beta\)-LIP, and C/EBP\(\beta\)-LAP are elevated (Fig. 2E) and are required for proper liver proliferation.
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LPS injections and inhibition of CaM. C/EBPβ was also included in these studies because it is a strong inhibitor of liver proliferation and it is reduced after LPS treatments similar to reduction after PH (5, 13). We found that levels of C/EBPβ isoforms are elevated at 24 h and slightly reduced at 48 h after LPS injections (Fig. 4, A and B). C/EBPα levels are reduced at 24 h but are returned to normal levels at 48 h.

We next inhibited expression CaM and examined liver proliferation after treatments of the mice with LPS. Liver proliferation was examined by measuring BrdUrd uptake. Fig. 4C shows that siRNA to CaM inhibits expression of CaM to 50–60%. Under these conditions livers of LPS-treated mice start proliferation and incorporate BrdUrd at 48 and 72 h after LPS treatments (Fig. 4, D and E). We found that up to 8–10% of hepatocytes proliferate in LPS-treated and siCaM-injected mice. To determine whether this proliferation is mediated by C/EBPβ isoforms, we simultaneously inhibited CaM and C/EBPβ by specific siRNAs. As one can see in Fig. 4C, siRNA to C/EBPβ almost completely inhibits expression of C/EBPβ-LIP and C/EBPβ-LAP isoforms. We found that the inhibition of C/EBPβ abolishes proliferation of the liver that was initiated by siRNA to CaM and LPS injections (Fig. 4, D and E). Thus, these studies showed that livers of LPS-treated mice do not proliferate due to CaM-mediated inhibition of C/EBPβ-LIP and perhaps C/EBPβ-LAP and that down-regulation of CaM is sufficient for the initiation of C/EBPβ-dependent liver proliferation.

Overexpression of C/EBPβ-LIP above CaM Levels Initiates Liver Proliferation in LPS-treated Mice—Because the endogenous C/EBPβ-LAP and C/EBPβ-LIP are expressed from the same mRNA, it is not possible to inhibit C/EBPβ-LIP without inhibition of C/EBPβ-LAP (see Fig. 4C). Therefore, the data with siRNA to C/EBPβ did not distinguish the contribution of C/EBPβ-LIP and C/EBPβ-LAP in the promotion of liver proliferation in mice with reduced levels of CaM. To examine the role of C/EBPβ-LIP in liver proliferation after LPS injection, we overexpressed FLAG-C/EBPβ-LIP above CaM levels and examined if this might promote liver proliferation under conditions of APR. FLAG-C/EBPβ-LIP was injected in mice as described under “Experimental Procedures,” and animals were treated with LPS for 24, 48, and 72 h. It is known that C/EBPβ-LIP affects the C/EBPα and C/EBPβ-LAP activities through dimerization as well as via repression of the C/EBPα promoter (6, 13). Therefore, we initially calculated levels of C/EBPβ-LAP and C/EBPα in livers of mice injected with FLAG-C/EBPβ-LIP. Western blotting analyses showed that FLAG-C/EBPβ-LIP is expressed at high levels at all examined time points. Calculations of ratios of total LIP (FLAG-C/EBPβ-LIP above levels of CaM and C/EBPβ-LAP) and C/EBPβ-LAP activities through dimerization as well as via repression of the C/EBPα promoter revealed that these ratios are 6–8-fold higher in FLAG-C/EBPβ-LIP injected livers compared to control livers at all examined time points. These studies showed that the injections of FLAG-C/EBPβ-LIP resulted in the significant excess of C/EBPβ-LIP above levels of CaM and C/EBPβ-LAP. It has been previously shown that C/EBPβ-LIP directly binds to C/EBPα promoter and represses expression of C/EBPα in the liver (13). Therefore, we have examined if the injected FLAG-C/EBPβ-LIP is biologically active by measuring levels of C/EBPα. We found that FLAG-C/EBPβ-LIP inhibits expression of C/EBPα (Fig. 5A). These studies revealed that injection of FLAG-C/EBPβ-LIP resulted in the elevation of protein levels of C/EBPβ-LIP to levels that are above CaM and that FLAG-C/EBPβ-LIP is biologically active and inhibits expression of C/EBPα. Examination of BrdUrd uptake in these mice showed that up to 5–6% of hepatocyte are BrdUrd-positive in LPS-treated and FLAG-C/EBPβ-LIP-injected mice, whereas no BrdUrd uptake is detected in LPS-treated mice that were transfected with an empty vector (Fig. 5, B and C). Thus, these studies show that overexpression of C/EBPβ-LIP above levels of CaM in LPS treated mice is sufficient to initiate liver proliferation.
C/EBPβ-LIP Promotes Proliferation of HEK293 Cells—Examination of the mechanisms of C/EBPβ-LIP-mediated cell proliferation in the liver is complicated because it is not possible to distinguish C/EBPβ-LIP from C/EBPβ-LAP. Therefore, we performed a search for tissue culture systems in which C/EBPβ-LIP accelerates proliferation. For these studies, C/EBPβ-LIP was cloned into pAdTrack vector, which also expresses green fluorescent protein from an independent cytomegalovirus promoter; therefore, each transfected cell expresses C/EBPβ-LIP and green fluorescent protein (see Fig. 6A). To screen cultured cells for the ability of C/EBPβ-LIP to promote cell proliferation, we used colony formation assay. In this assay cells are transfected at very low density so that there are only single cells on the plates in the beginning of experiments. In several days the proliferating cells form cell clusters containing two and more cells, whereas inhibited cells stay as single cells. A typical picture of green colonies is shown in Fig. 6A. We found that HEK293 and Hep3B2 cells have quite different responses to the ectopic expression of C/EBPβ-LIP. As one can see in Fig. 6B, ectopic expression of C/EBPβ-LIP in HEK293 cells significantly increases the amounts of proliferating cells at day 4; however, the significant portion of Hep3B2 cells transfected with C/EBPβ-LIP does not proliferate and is rather inhibited by C/EBPβ-LIP. The comparison of proliferating cells transfected with an empty vector and with C/EBPβ-LIP showed that C/EBPβ-LIP accelerates proliferation of HEK293 cells (Fig. 6C). Given the established cell line for the growth promotion activities of C/EBPβ-LIP, we asked if CaM might block the activity of C/EBPβ-LIP. We have co-transfected CaM with C/EBPβ-LIP and found that CaM abolishes the growth promotion activity of C/EBPβ-LIP (Fig. 6D). This effect of CaM is mediated through direct interactions with C/EBPβ-LIP as the CaMΔ mutant is not able to reduce growth promotion activities of C/EBPβ-LIP.
HEK293 Cells Contain Abundant Rb-E2F1 Complex—Because C/EBPα-LIP preferentially interacts with Rb in proliferating livers (Fig. 1), we suggested that the different biological activities of C/EBPα-LIP in HEK293 and Hep3B2 cells might be associated with differences in expression of E2F-Rb complexes. We found that the amounts of Rb are higher in HEK293 cells than in Hep3B2 cells and that electrophoretic mobility of Rb differs in these cells (Fig. 7A), suggesting that Rb might be differentially phosphorylated. Although the phosphorylation of Rb leads to dissociation of E2F-Rb complexes (37, 38), Inoue et al. (39) found that phosphorylation of Rb at Ser-612 does not block its interactions with E2F1 and that Ser-612-ph-Rb/E2F1 complexes repress the E2F-dependent promoters. Western blotting analysis with phospho-specific Abs of Rb showed that the Rb-Ser-612-ph isoform is abundant in HEK293 cells, but it is not detectable in Hep3B2 cells. Examination of another isoform of Rb, Ser-811-ph, showed that the amounts of this isoform are identical in the tested cells. We also found that expression of E2F1 and E2F4 is higher in HEK293 cells compared with Hep3B2 cells. Examination of CaM showed approximately identical levels of CaM in HEK293 and Hep3B2 cells.

We next examined the compositions of E2F-Rb complexes in HEK293 and in Hep3B2 cells by EMSA approach. Three major complexes were detected in HEK293 cells (Fig. 7B). Incorporation of specific antibodies into the binding reactions revealed that the slower migrating band represents the E2F1-Rb complex. Because Rb is phosphorylated at Ser-612 in HEK293 cells, we asked if this isoform of Rb is involved in the formation of Rb-E2F1 complexes. EMSA showed that Abs to Ser-612-ph supershifted the E2F1-Rb complex (Fig. 7C). Quite different compositions of the Rb-E2F complexes are observed in Hep3B2 cells (Fig. 7D). In these cells free E2F4 and E2F4p130 represent the major complexes that interact with the DHFR probe. Taken together, these studies show that HEK293 and Hep3B2 cells express different levels of E2F, Rb, and p130 proteins and contain different E2F-Rb complexes.

C/EBPα-LIP Activates the B-myb Promoter by a Release of Rb-dependent Repression of the Promoter—Given the interaction of C/EBPβ-LIP with Rb (Fig. 2), we suggested that C/EBPβ-LIP might activate E2F-dependent promoters that are repressed by Rb. B-myb promoter was used to test this suggestion as it has been shown to be repressed by Rb-E2F complexes (37). The WT and mutant B-myb promoters (Fig. 8A) were co-transfected with C/EBPβ-LIP and with an empty vector into HEK293 cells. The cells were starved for 48 h, and the activity of the promoters was examined. We found that expression of
C/EBPβ-LIP causes activation of the WT B-myb promoter. This activation is mediated via elimination of Rb-E2F-dependent repression as C/EBPβ-LIP does not change the activity of the mutant B-myb promoter, which is no longer under control of the Rb-E2F complexes. Thus, these studies showed that C/EBPβ-LIP activates the B-myb promoter by the release of Rb-mediated repression. We have next examined if CaM inhibits this activity of C/EBPβ-LIP. We have co-transfected WT CaM and the mutant CaM with C/EBPβ-LIP into HEK293 cells. These studies showed that WT CaM blocks C/EBPβ-LIP-mediated release of the repression of the B-myb promoter, whereas the mutant CaM does not change the C/EBPβ-LIP-mediated de-repression of the promoter. Because all three members of Rb family might repress the B-Myb promoter (37, 38), we have performed the second set of experiments in which we examined if the competition between CaM and Rb regulates the E2F-dependent promoters. These studies showed that ectopic expression of Rb in HEK293 cells further represses the B-myb promoter and that C/EBPβ-LIP releases this repression. WT CaM blocks the ability of C/EBPβ-LIP to release Rb-mediated repression of the B-myb promoter; however, the CaMΔ mutant is not able to do this.

FIGURE 6. Ectopic expression of C/EBPβ-LIP promotes proliferation of HEK293 cells. A, shown is the structure of pAdTrack-C/EBPβ-LIP plasmid (upper) and a typical picture of the colony growth assay (see “Experimental Procedures” for more details). GFP, green fluorescent protein; CMV, cytomegalovirus. B, ectopic expression of C/EBPβ-LIP promotes proliferation of HEK293 cells. pAdTrack-C/EBPβ-LIP was transfected into HEK293 and Hep3B2; cells and the proliferation of green cells were examined by colony formation assay at day 4 after transfections. Bar graphs show the results as a summary of three independent experiments. The percentage of proliferating cells is shown. V, vector. C, overexpression of CaM in HEK293 cells reduces the ability of C/EBPβ-LIP to promote cell proliferation. HEK293 cells were transfected with C/EBPβ-LIP, CaM, and with C/EBPβ-LIP + CaM. The percentage of proliferating cells is shown. Bar graphs represent a summary of three independent experiments. D, CaM blocks growth promotion activities of C/EBPβ-LIP via direct interactions. The experiments were performed as described above. C/EBPβ-LIP was co-transfected with WT CaM and with CaMΔ. The bottom image shows Western blotting with Abs to C/EBPβ-LIP using protein extracts isolated from the experimental cells. L, LIP, C, CaM.
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that both C/EBPα and bacterially expressed, purified to homogeneity C/EBPβ-LIP bind to all tested E2F-dependent promoters (Fig. 9D). We have next examined if CaM might inhibit the interaction of C/EBPβ-LIP with the E2F consensuses. Fig. 9E shows that WT CaM dramatically reduces the interactions of C/EBPβ-LIP with the DHFR promoter, whereas the mutant CaMΔ does not significantly affect the interactions of C/EBPβ-LIP with the DHFR promoter.

To examine if C/EBPβ-LIP interacts with E2F-dependent promoters in vivo and displaces E2F1-Rb complexes from the promoters, we performed chromatin-IP analysis. Because endogenous C/EBPβ-LIP cannot be distinguished from C/EBPβ-LAP by available antibodies, we transfected FLAG-tagged C/EBPβ-LIP alone or with WT CaM and CaMΔ into HEK293 cells and examined the occupation of endogenous B-myb and DHFR promoters by E2F1, Rb, and FLAG-C/EBPβ-LIP. In control cells B-myb and DHFR promoters are occupied by the Rb-E2F1 complex, which represses these promoters (Fig. 9F). The expression of FLAG-C/EBPβ-LIP causes a reduction of the complexes on the B-myb and DHFR promoters, whereas FLAG-C/EBPβ-LIP is abundant on the promoters, suggesting that it displaces the Rb-E2F1 complexes from the E2F-dependent promoters (Fig. 9F). WT CaM blocks the C/EBPβ-LIP-dependent displacements of Rb-E2F1 complexes, whereas the mutant CaMΔ is not able to block this activity of C/EBPβ-LIP (Fig. 9F). Thus, these studies show that C/EBPβ-

DISCUSSION

Liver is a unique tissue that is able to regenerate itself after surgical resections (1–4). The transition of the liver from quiescence to proliferation requires the orchestrated re-organization of a number of pathways including changes in activity of C/EBP family proteins (2, 4). C/EBPβ is expressed at high levels in the liver, and it is one of the critical regulators of liver growth and differentiation (5, 6). The biological functions of C/EBPβ-
LAP have been intensively investigated; however, very little is known about biological functions of the truncated isoform C/EBPβ-LIP. In this paper we have identified molecular mechanisms by which C/EBPβ-LIP promotes liver proliferation and mechanisms by which liver controls this activity. C/EBPβ-LIP is increased in several biological situations; however, its growth promotion activity is displayed only in livers after partial hepatectomy. In searching for the proteins that might control activities of C/EBPβ-LIP, we have identified CaM as a protein that interacts with C/EBPβ and blocks growth promotion activities of C/EBPβ-LIP. CaM has been previously implicated in the translation of calcium signaling mainly through four mechanisms (19). Data in our paper suggest an additional mechanism by which CaM translates calcium signaling. The reduction of Ca²⁺ after LPS treatment leads to the increased interactions of CaM with C/EBPβ-LIP in the liver. It is important to note that previous studies have shown that treatment of rats with LPS increases intracellular calcium in Kupffer cells and in endothelial cells but not in the hepatocytes (40). In addition, the LPS-mediated elevation of calcium in cultured hepatic microphages.
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that CaM might regulate cell cycle progression through interactions with p21 and with cyclin E in other tissues (24, 25, 28). It would be interesting to examine if CaM interacts with these proteins in the liver and if these possible interactions might be involved in the regulation of liver proliferation. Because Ca\(^{2+}\) regulates interactions of CaM and C/EBP\(^{\beta}\), we suggest that CaM is one of the important regulators of liver proliferation as a mediator of calcium signaling.

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REFERENCES

1. Diehl, A. M. (2005) Am. J. Physiol. Gastrointest. Liver Physiol. 288, G1–G6
2. Fausto, N., Campbell, J. S., and Riehle, J. K. (2006) Hepatology 43, 545–555
3. Michalopoulous, G. K. (2007) J. Cell. Physiol. 213, 286–300
4. Timchenko, N. A. (2009) Trends Endocrinol. Metab. 20, 171–176
5. Greenbaum, L. E., Li, W., Cressman, D. E., Peng, Y., Ciliberto, G., Poli, V., and Taub, R. (1998) J. Clin. Invest. 102, 996–1007
6. Johnson, P. F. (2005) J. Cell Sci. 118, 2545–2555
7. Calkhoven, C. F., Müller, C., and Leutz, A. (2000) Genes Dev. 14, 1920–1932
8. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
9. Hsieh, C. C., Xiong, W., Xie, Q., Rabek, J. P., Scott, S. G., An, M. R., Reisner, P. D., Kuninger, D. T., and Papavassiliou, J. (1998) Mol. Biol. Cell 9, 1479–1494
10. Harrison, J. R., Huang, Y. F., Wilson, K. A., Kelly, P. L., Adams, D. J., Gronowicz, G. A., and Clark, S. H. (2005) J. Biol. Chem. 280, 8117–8124
11. Hata, K., Nishimura, R., Ueda, M., Ikeda, F., Matsubara, T., Ichida, F., Hisada, K., Nokubi, T., Yamaguchi, A., and Yoneda, T. (2005) Mol. Cell. Biol. 25, 1971–1979
12. Timchenko, N. A., Welm, A. L., Lu, X., and Timchenko, L. T. (1999) Nucleic Acids Res. 27, 4517–4525
13. Welm, A. L., Mackey, S. L., Timchenko, L. T., Darlington, G. J., and Timchenko, N. A. (2000) J. Biol. Chem. 275, 27406–27413
14. Timchenko, L. T., Salisbury, E., Wang, G. L., Nguyen, H., Albrecht, J. H., Hershey, J. W., and Timchenko, N. A. (2006) J. Biol. Chem. 281, 32806–32819
15. Timchenko, N. A., Wang, G. L., and Timchenko, L. T. (2005) J. Biol. Chem. 280, 20549–20557
16. Baldwin, B. R., Timchenko, N. A., and Zahnower, C. A. (2004) Mol. Cell. Biol. 24, 3682–3691
17. Zahnower, C. A. (2009) Expert Rev. Mol. Med. 11, e12
18. Luedde, T., Duderstadt, M., Streetz, K. L., Tacke, F., Kubicka, S., Manns, M. P., and Trautwein, C. (2004) Hepatology 40, 356–365
19. Liu, J. O. (2009) Immunol. Rev. 228, 184–198
20. Swulius, M. T., and Waxham, M. N. (2008) Cell. Mol. Life Sci. 65, 2637–2657
21. Wayman, G. A., Lee, Y. S., Tokunitsu, H., Silva, A. J., Silva, A., and Soderling, T. R. (2008) Neuron 59, 914–931
22. Finkler, A., Ashery-Padan, R., and Fromm, H. (2007) FEBS Lett. 581, 3893–3898
23. Monaco, S., Illario, M., Rusciano, M. M., Gragnaniello, G., Spigna, G. D., Leggiiero, E., Pastore, L., Feni, G., Rossi, G., and Vitale, M. (2009) Cell Cycle 8, 2024–2030
24. Choi, J., Chiang, A., Taulier, N., Gros, R., Pirani, A., and Husain, M. A. (2006) Circ. Res. 98, 1273–1281
25. Choi, J., and Husain, M. (2006) Cell Cycle 5, 2183–2186
26. Koledova, V. V., and Khalil, R. A. (2006) Circ. Res. 98, 1240–1243
27. Rodríguez-Villarrubia, A., Jaumot, M., Abella, N., Canela, N., Brun, S., Díaz, C., Estanyol, J. M., Bachs, O., and Agell, N. (2005) Mol. Cell. Biol. 25, 7364–7374
28. Taulés, M., Rodríguez-Villarrubia, A., Rius, E., Estanyol, J. M., Casanova,
O., Sacks, D. B., Pérez-Payá, E., Bachs, O., and Agell, N. (1999) J. Biol. Chem. 274, 24445–24448
29. Conboy, I. M., Conboy, M. J., Wagers, A. I., Girma, E. R., Weisman, I. L., and Rando, T. A. (2005) Nature 433, 760–764
30. Iakova, P., Awad, S. S., and Timchenko, N. A. (2003) Cell 113, 495–506
31. Wang, G. L., and Timchenko, N. A. (2005) Mol. Cell. Biol. 25, 1325–1338
32. Chang, H. T., Huang, C. C., Cheng, H. H., Wang, J. L., Lin, K. L., Hsu, P. T., Tsai, J. Y., Liao, W. C., Lu, Y. C., Huang, J. K., and Jan, C. R. (2008) Toxicol. Lett. 179, 53–58
33. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) J. Cell Biol. 105, 2145–2155
34. Charles, A., Tang, X., Crouch, E., Brody, J. S., and Xiao, Z. X. (2001) J. Cell. Biochem. 83, 414–425
35. Chen, P. L., Riley, D. J., Chen, Y., and Lee, W. H. (1996) Genes Dev. 10, 2794–2804
36. Chen, P. L., Riley, D. J., Chen-Kiang, S., and Lee, W. H. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 465–469
37. Dyson, N. (1998) Genes Dev. 12, 2245–2262
38. Van den Heuvel, S., and Dyson, N. J. (2008) Nat. Res. Mol. Cell. Biol. 9, 713–724
39. Inoue, Y., Kitagawa, M., and Taya, Y. (2007) EMBO J. 26, 2083–2093
40. Portolés, M. T., Arahuetes, R. M., and Pagani, R. (1994) Eur. J. Cell Biol. 65, 200–205
41. Enomoto, N., Yamashina, S., Goto, M., Schemmer, P., and Thurman, R. G. (1999) Am. J. Physiol. 277, G1251–G1258