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Aging Reduces Proliferative Capacities of Liver by Switching Pathways of C/EBPα Growth Arrest

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Summary

The liver is capable of completely regenerating itself in response to injury and after partial hepatectomy. In liver of old animals, the proliferative response is dramatically reduced, the mechanism for which is unknown. The liver specific protein, C/EBPα, normally arrests proliferation of hepatocytes through inhibiting cyclin dependent kinases (cdks). We present evidence that aging switches the liver-specific pathway of C/EBPα growth arrest to repression of E2F transcription. We identified an age-specific C/EBPα-Rb-E2F4 complex that binds to E2F-dependent promoters and represses these genes. The C/EBPα-Rb-E2F4 complex occupies the c-*myc* promoter and blocks induction of c-*myc* in livers of old animals after partial hepatectomy. Our results show that the age-dependent switch from cdk inhibition to repression of E2F transcription causes a loss of proliferative response in the liver because of an inability to induce E2F target genes after partial hepatectomy providing a possible mechanism for the age-dependent loss of liver regenerative capacity.

Introduction

The removal of a portion of liver (partial hepatectomy, PH) leads to initiation of liver proliferation and to a final restoration of the original mass (Fausto and Webber, 1994). It has been shown that livers of old animals have a reduced proliferative response, which is characterized by a significant delay in cell cycle entry and by a significant reduction of the number of proliferating hepatocytes (Bucher et al., 1964; Fry et al., 1984; Timchenko et al., 1998). A molecular basis for the loss of proliferative capacity in old livers has not been identified. Liver proliferation after PH requires an orchestrated cascade of gene expression, which includes: (1) the activation of a number of transcription factors, (2) the induction of proteins that drive cell cycle progression, and (3) downregulation of proteins that inhibit cell proliferation (Fausto, 2000; Taub, 1996). At very early stages of liver proliferation initiated by PH, the transcription factors Stat-3, NF-kB, and C/EBPβ are activated mainly through posttranslational modifications (Taub, 1996). Then, the temporary induction of cyclins D1, E, and A activates cdk4 and cdk2. Cdk2 and cdk4 phosphorylate Rb family proteins, leading to a release of E2F transcription factors followed by activation of S phase and mitotic specific genes by E2F (Fausto, 2000).

In addition to these events, several growth inhibitory proteins must be reduced after PH to allow cell proliferation. One of these proteins, a member of the bZIP family of transcription factors C/EBPα, is expressed at high levels in liver (Birkenmeier et al., 1989) and maintains liver quiescence (Wang et al., 2001; Timchenko et al., 1997). Livers of newborn C/EBPα knockout mice have an increased rate of proliferation, demonstrating that C/EBPα is required for the inhibition of liver proliferation (Timchenko et al., 1997). Molecular mechanisms of C/EBPα-mediated growth arrest have been the subject of intensive investigations during the last ten years; however, only recent observations shed light on some aspects of the growth inhibition. Numerous attempts to identify cell cycle-related transcriptional targets of C/EBPα were unsuccessful. It was recently demonstrated that although C/EBPα is a transcription factor, its growth inhibitory activity in liver of young animals is mediated through direct interaction with cdks (Wang et al., 2001, 2002). In adipose and myeloid tissues, the antiproliferative effect of C/EBPα is mediated through repression of E2F-dependent transcription (Porse et al., 2001). The block of C/EBPα-dependent repression of E2F-target genes causes an adipose hypoplasia and myeloid dysplasia, but does not affect liver functions (Porse et al., 2001) showing that the pathway of C/EBPα growth arrest in the liver differs from that operating in adipose tissues.

In this paper, we present evidence that aging switches the C/EBPα pathway of growth arrest in liver from cdk inhibition to repression of E2F transcription. Aging increases levels of a chromatin remodeling protein Brm, which in turn interacts with C/EBPα and initiates the formation of a high MW complex: C/EBPα-Rb-E2F4. This age-specific C/EBPα-Rb-E2F4 complex binds to promoters regulated by E2F and represses expression of these genes in quiescent and regenerating livers of old animals. We present evidence that the C/EBPα-Rb-E2F4 complex blocks the activation of the c-*myc* promoter in old livers after PH and in tissue culture models. Our data provide a molecular basis for the reduced proliferative response in old livers; the switch of the C/EBPα growth arrest pathway from inhibition of cdks to repression of E2F transcription leads to a failure of the old livers to eliminate the growth inhibitory activity of C/EBPα.

Results

Old Animals Contain a High Molecular Weight C/EBPα-Rb-E2F4-Brm Complex

We have shown that the loss of proliferative response in old livers correlates with a failure to reduce protein levels of C/EBPα (Timchenko et al., 1998). Because C/EBPα inhibits cell proliferation through protein-protein interactions (Wang et al., 2001), we suggested that the high levels of C/EBPα might interfere with cell cycle

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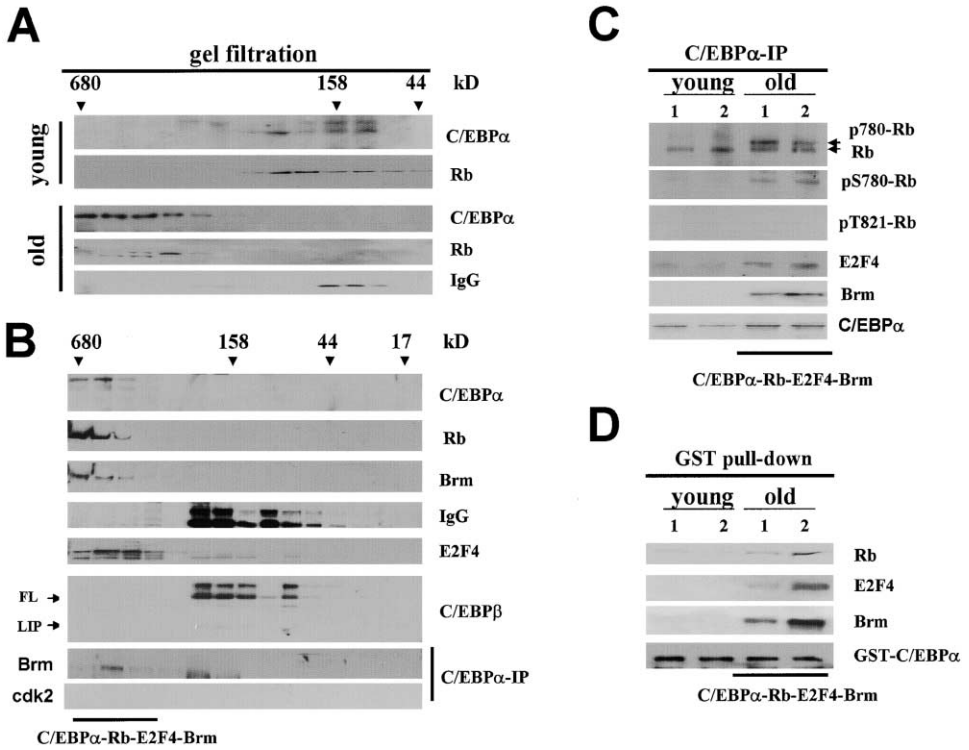

Figure 1. The Majority of C/EBPα in Livers of Old Animals Exists as C/EBPα-Rb-E2F4-Brm Complex

(A) C/EBPα forms high molecular mass complexes in livers of old animals. Nuclear extracts from young and old animals were separated by gel filtration. The presence of C/EBPα and Rb in gel-filtration fractions was examined by Western blotting. IgG, Western blotting with secondary antibodies to IgG to detect the position of heavy chains of IgGs.

(B) C/EBPα-Rb-E2F4-Brm complex is abundant in old livers. Nuclear extracts from livers of old animals were fractionated by gel filtration and the fractions were examined by Western blotting with antibodies to C/EBPα, Rb, Brm, E2F4, and C/EBPβ. Positions of C/EBPβ isoforms, LAP and LIP, are shown on the left. Bottom shows Western blotting of C/EBPα immunoprecipitates with antibodies to Brm and cdk2.

(C) Coimmunoprecipitation (C/EBPα-IP) assay with protein extracts from young and old animals. C/EBPα was precipitated from nuclear extracts of young and old mice. Rb, E2F4, Brm and C/EBPα were examined by Western blotting with specific antibodies. Data with two animals of each age group are shown.

(D) GST pull-down. Nuclear extracts from young and old mice were incubated with GST-C/EBPα. GST pull-down samples were analyzed by Western blotting with antibodies shown on the right.

progression through its interaction with cell cycle proteins. We examined protein-protein interactions of C/EBPα in livers of old rats and compared them with that observed in livers of young animals. HPLC-based gel-filtration technique was applied for these studies. Figure 1A shows a reproducible result of the gel-filtration assay of proteins from livers of young and old rats. In young animals, C/EBPα is observed in fractions with molecular weight ranging from 100 to 300 kDa, while in nuclear extracts of old animals C/EBPα is located in gel-filtration fractions with MW ranging from 400 kDa to 680 kDa (Figure 1A). Treatment of the nuclear extracts with DNase I before the fractionation does not alter the location of C/EBPα in gel-filtration fractions (data not shown) suggesting that the shift of C/EBPα into higher MW gel-filtration fractions is mediated by interactions with other proteins.

In addition to cdk2 and cdk4, C/EBPα interacts with Rb family proteins (Chen et al., 1996; Timchenko et al., 1999a), E2F transcription factors (Johansen et al., 2001), and with a chromatin remodeling protein Brm (Pedersen et al., 2001). Therefore, we examined whether these proteins colocalize with C/EBPα in the high MW gel-filtration fractions. In old animals, we observed colocalization of C/EBPα, E2F4, Rb, and Brm in high MW fractions, while in young animals C/EBPα does not colocalize with these proteins in high MW fractions (Figures 1A and 1B). The colocalization of C/EBPα with these proteins is specific for C/EBPα, since another member of C/EBP family, C/EBPβ, is observed only in low MW fractions (Figure 1B). To examine whether the colocalization of E2F4, Rb, and Brm with C/EBPα in high MW fractions is due to direct interactions, IP-Western experiments with the gel-filtration fractions were performed. As can be seen in Figure 1B, Brm is bound to C/EBPα, suggesting the existence of C/EBPα-Brm complexes. We also detected association of Rb with C/EBPα in high MW fractions (data not shown and see Figure 4). We have previously found that, in young livers, C/EBPα associates with cdk2 (Wang et al., 2001). The analysis of cdk2 in C/EBPα IPs from gel-filtration fractions showed that C/EBPα is not associated with cdk2 in old livers (Figure 1B, bottom). We have performed the gel-filtration analysis of 6 livers of 24-month-old rats and 7 livers of 22–24-month-old mice and equal numbers of young (6–8 months) rats and young (4–6 months) mice. In livers of

old animals, we reproducibly observed the formation of the age-specific complex.

To further characterize this complex, we applied a simple coimmunoprecipitation procedure. As can be seen in Figure 1C, amounts of all of these proteins are increased in C/EBPα IPs from old animals. In addition to the increase of total amounts of Rb associated with C/EBPα, we also observed the enrichment of hyperphosphorylated forms of Rb in C/EBPα IPs from old animals (Figure 1C). The analysis of C/EBPα IPs with antibodies specific to ph-Ser-780 (cyclinD/cdk4 phosphorylation site) and with antibodies to ph-T-821 (cyclin E-cdk2 phosphorylation site) showed that the phosphorylated form of Rb contains ph-Ser-780. In agreement with Co-IP results, GST-pull-down assay shows that Brm, Rb, and E2F4 all interact with GST-C/EBPα in extracts from old animals better than in extracts from young animals (Figure 1D). Thus, gel filtration, Co-IP, and GST-pull-down assays show that the major portion of C/EBPα in old animals exists in a complex with Rb, E2F4, and Brm.

Induction of Brm in Livers of Old Animals Leads to the Appearance of the C/EBPα-Rb-E2F4-Brm Complex

We next investigated the mechanism that leads to the increase of C/EBPα-Rb-E2F4-Brm complex in livers of old animals. First, we examined total levels of all components by Western blotting. A representative result of these studies is shown in Figure 2A. Protein levels of C/EBPα, Rb, and E2F4 are similar in quiescent livers of young and old animals. However, protein levels of Brm are significantly increased in livers of old animals. Calculations of Brm levels as a ratio to β actin show a 3- to 4-fold induction in livers of old rats and a 4-to 5-fold induction in livers of old mice (Figure 2B). Given that Brm and a highly homologous protein Brg1 are redundant (Reyes et al., 1998), we also examined protein levels of Brg1 in young and old livers. Western blotting shows that Brg1 levels are not changed in old livers (Figure 2A). Because the increase of Brm expression correlates with the appearance of the age specific C/EBPα complex, we suggested that the elevation of Brm is responsible for the appearance of the complex in old livers. To test this suggestion, we purified mouse Brm protein from livers of old animals using a combination of HPLC-based chromatography columns (see Experimental Procedures). Figure 2C shows the analysis of C/EBPα complexes in young livers before and after incubation with Brm. In agreement with previous observations, C/EBPα is located in the regions ranging from 100 to 300 kDa in extracts from young animals (Wang et al., 2001). In young livers, C/EBPα forms complexes with cdks since cdk2 is detected in C/EBPα precipitates (Figure 2C, upper image), while no Brm is observed in C/EBPα IPs. After the incubation of the nuclear extracts with Brm, C/EBPα, Rb, and E2F4 are shifted to fractions containing high molecular weight complexes. Immunoprecipitation of C/EBPα and Western blot with antibodies to Rb revealed that this shift is due to the formation of the C/EBPα-Rb-E2F4-Brm complex (Figure 2C, bottom). Although in young livers C/EBPα is associated with cdk2, cdk2 is not detected in C/EBPα IPs after incubation of nuclear extracts with Brm. Given the fact that cdk2 and Brm interact with the same region of C/EBPα (Wang et al., 2001, and see Figure 3B), we suggested that Brm might replace cdk2 from C/EBPα. To examine this suggestion, we performed a direct test whether these proteins compete for the interaction with C/EBPα. Full-length GST-C/EBPα was incubated with nuclear extracts from young animals in the presence of increasing amounts of Brm. These studies revealed that Brm replaces cdk2 from C/EBPα (Figure 2D). A similar result was obtained with a short C/EBPα fragment (aa 140–207) to which both proteins bind (Figure 2D, right). Thus, these studies demonstrate that increased levels of Brm replace cdk2 from C/EBPα and lead to the formation of the age-specific C/EBPα-Rb-E2F4-Brm complex (Figure 2E).

To better characterize the role of Brm in the formation of the age-specific complex, we investigated whether the C/EBPα-Rb-E2F4-Brm complex functions in differentiated 3T3-L1 adipocytes and whether Brm controls the formation of the complex in these cells. We chose 3T3-L1 adipocytes because Porse et al. (2001) recently reported that C/EBPα causes growth arrest in adipose tissues via repression of E2F transcription. In 3T3-L1 cells, C/EBPα is a key regulator of growth arrest and differentiation (Umek et al., 1991; Yeh et al., 1995). The differentiation of 3T3-L1 cells was initiated as described (Timchenko et al., 1999b). Protein levels of C/EBPα and Brm are increased in differentiated adipocytes, while levels of Rb are not altered (Figure 3A, Western blot). E2F4 levels are slightly induced at day 1 and returned to original levels in differentiated cells and E2F1 levels are not changed during differentiation (Figure 3A). The induction of Brm and C/EBPα leads to the formation of the C/EBPα-Rb-E2F4-Brm complex since all these proteins are present in C/EBPα IPs. To further characterize the complex, proteins from differentiated adipocytes (day 6) were fractionated by gel filtration and the complex was analyzed as described above. All components of the complex are colocalized in high MW fractions and form the C/EBPα-Rb-E2F4-Brm complex since Brm is observed in C/EBPα IPs from high MW fractions (Figure 3B). Thus, the analysis of protein-protein complexes of C/EBPα in 3T3-L1 adipocytes shows that, similar to livers of old animals, the induction of Brm leads to the formation of the complex. Taken together, these investigations demonstrate that the induction of Brm is sufficient to cause the formation of the age-specific complex in vitro, in old livers and in 3T3-L1 adipocytes during differentiation.

The Formation of C/EBPα-Rb-E2F4-Brm Complex Requires Functional Rb and Two Regions of C/EBPα

Next, we determined the region(s) of C/EBPα, which interact with the components of the complex. GST pull-down assay was performed with constructs shown in Figure 3C. It has been previously demonstrated that a region of C/EBPα between aa 50 and 95 interacts with Rb family proteins (Chen et al., 1996; Timchenko et al., 1999a; Porse et al., 2001), and that another region of C/EBPα, between aa 120 and 200, interacts with Brm (Pedersen et al., 2001). In agreement with these data,

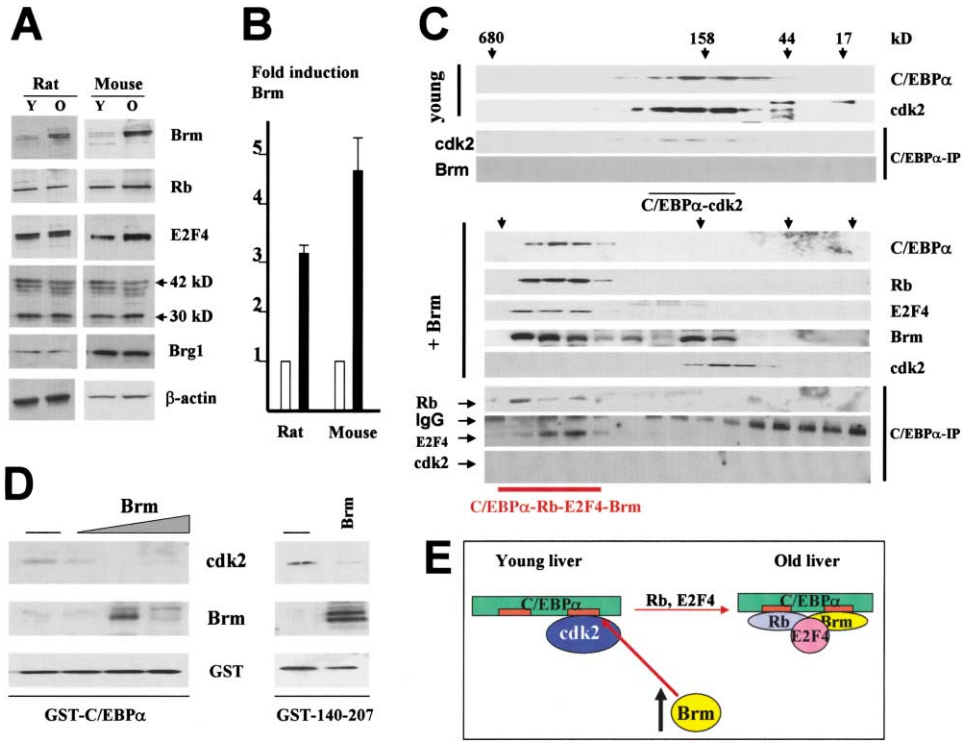

Figure 2. Induction of Brm Levels in Old Livers Leads to the Appearance of the C/EBPα-Rb-E2F4-Brm Complex

(A) Protein levels of Brm are increased in old animals. Western blotting of nuclear extracts from rat and mouse livers was performed with antibodies shown on the right. Two isoforms of C/EBPα (42 kDa and 30 kDa) are shown by arrows.

(B) Brm levels were calculated as a ratio to β actin. Summary of five independent experiments with three animals of each age group is shown.

(C) Addition of an excess of Brm to nuclear proteins from young animals leads to the formation of C/EBPα-Rb-E2F4-Brm complex. Upper image, nuclear extracts from young livers were fractionated by gel filtration. C/EBPα and cdk2 were examined in each fraction by Western blotting. C/EBPα was precipitated from gel-filtration fractions, and Brm and cdk2 were determined in C/EBPα IPs by Western blotting. Bottom image, 2 μg of purified Brm was incubated with nuclear proteins (600 μg) from young livers. Proteins were fractionated by gel filtration, and fractions were analyzed by Western blotting as described in the legend to Figure 1. Bottom image shows Western analysis of C/EBPα immunoprecipitates from gel-filtration fractions with antibodies to Rb, E2F4, and cdk2.

(D) Brm replaces cdk2 from C/EBPα molecule. GST-C/EBPα or GST-C/EBPα-140-207 were incubated with nuclear extracts from young livers in the presence of increasing amounts of Brm, washed with PBS, and analyzed by Western blotting with antibodies to cdk2, Brm, and GST.

(E) A model for the appearance of the age-specific C/EBPα-Rb-E2F4-Brm complex. Proteins levels of Brm are increased in old livers leading to the replacement of cdk2 from C/EBPα and to the formation of the C/EBPα-Rb-E2F4-Brm complex.

GST-pull-down assay indicates that C/EBPα-2 and Δ113 constructs, containing both these regions, interact with Brm, Rb, E2F1, and E2F4 proteins (Figure 3D). None of these proteins interacts with the short 50 amino acid N terminus of C/EBPα. Deletion of Rb interacting region (construct ΔDLF) or Brm interacting region (construct N142) results in the failure of C/EBPα to interact with E2F1 or E2F4. However, the interaction of C/EBPα with Rb and Brm is still observed with both deletion constructs. These data are consistent with observations that Rb independently interacts with Brm (Dunaief et al., 1994; Strober et al., 1996), suggesting that the Rb or Brm interacting regions alone can pull down a Brm-Rb-C/EBPα complex. Thus, these studies demonstrated that the formation of the age-specific C/EBPα-Rb-E2F4-Brm complex requires the interaction of C/EBPα with both Rb and Brm proteins.

We next examined whether Rb is required for the formation of C/EBPα-Rb-E2F4-Brm complex. For this goal, we generated a stable clonal cell line with Rb-deficient SAOS2 cells (clone SA12, see Figure 6), in which C/EBPα was cloned under Lac-repressor control. Western analysis showed that Brm and E2F4 are expressed at high levels in glucose-treated cells (control) and in IPTG-treated cells where C/EBPα is induced (Figure 3E). Co-IP studies demonstrate that although C/EBPα interacts with Brm, E2F1, and E2F4 are not detectable in C/EBPα IPs from cells that do not express Rb. These data are consistent with GST-pull-down results showing that the deletion of Rb interacting region abolishes the interaction of C/EBPα with E2F4 and E2F1. Taken together, these data show that two regions of C/EBPα are required for the formation of the age-specific C/EBPα complex and that the formation of the complex also requires functional Rb (Figure 3F).

C/EBPα-Rb-E2F4-Brm Complex Binds to E2F Consensuses In Vitro and Occupies E2F-Dependent Promoters in Livers of Old Animals

We next examined whether the age-specific C/EBPα complex interacts with DNA. Since the complex contains two transcription factors, C/EBPα and E2F4 (Figure 3F), it might potentially bind to both E2F and C/EBP dependent promoters. We first applied gel-shift assay to exam-

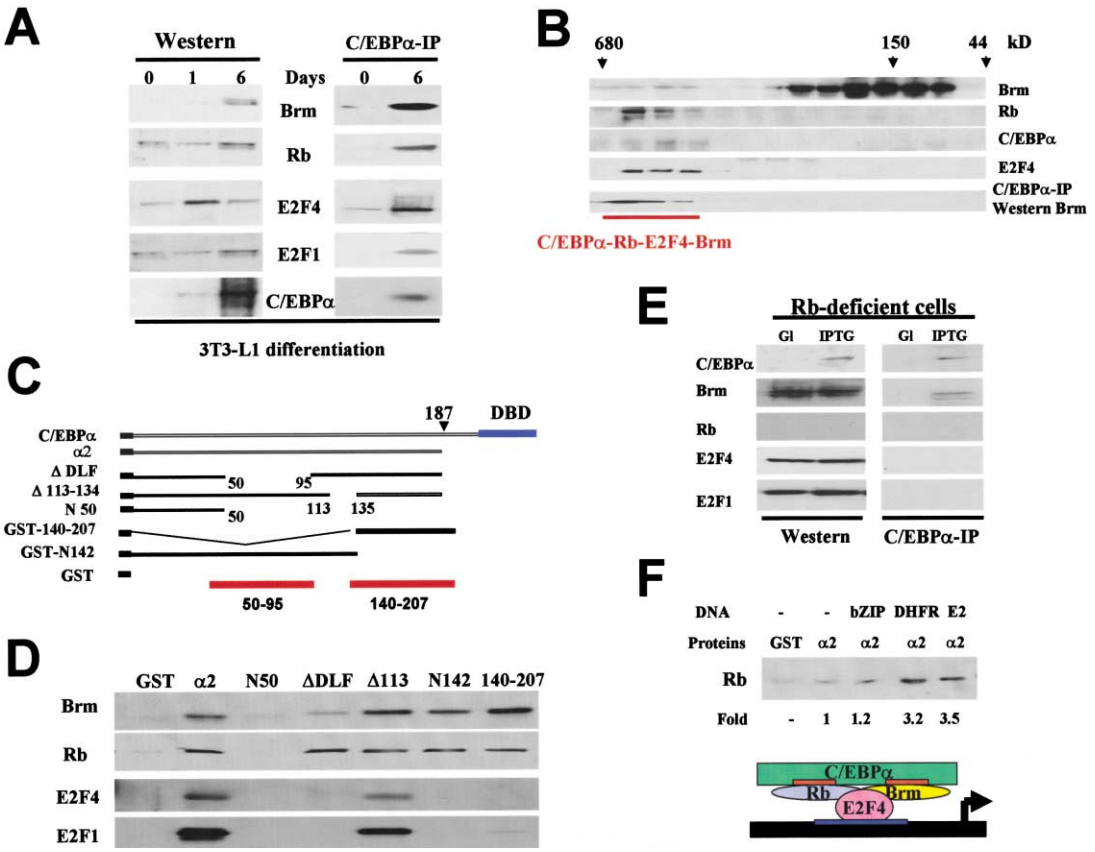

Figure 3. The C/EBPα-Rb-E2F4-Brm Complex Is Formed in Differentiated Adipocytes by Induction of Brm and Requires Functional Rb

(A) The induction of Brm in differentiated adipocytes leads to the formation of C/EBPα-Rb-E2F4-Brm complex. Western blot: expression of C/EBPα, Rb, Brm, E2F1, and E2F4 was examined in predifferentiated adipocytes (day 0), during mitotic expansion (day 1) and in differentiated (day 6) adipocytes by Western blotting. C/EBPα-IP: C/EBPα was immunoprecipitated from protein extracts (days 0 and 6). The presence of Brm, Rb, E2F1, and E2F4 in C/EBPα IPs was examined by Western blotting.

(B) Gel-filtration analysis of C/EBPα complexes in differentiated 3T3-L1 adipocytes (day 6). The experiment was performed as described in the legend to Figure 1.

(C) C/EBPα constructs used for GST pull-down assay. Red boxes show regions of C/EBPα that are involved in the formation of the C/EBPα-Rb-E2F4-Brm complex.

(D) Two regions of C/EBPα are required for the formation of C/EBPα-Rb-E2F4-Brm complex. Nuclear extracts from old animals were incubated with GST-C/EBPα constructs (shown on the top), and proteins (shown on the left) were examined by Western blotting.

(E) C/EBPα does not interact with E2F1 and E2F4 in cells lacking functional Rb. Protein extracts from Rb-deficient clone SA12 (see Figure 6) were examined by Western blotting with antibodies shown on the left. GI, control cells treated with glucose. IPTG, cells expressing C/EBPα (see Figure 6). C/EBPα was precipitated with specific antibodies, and C/EBPα IPs were examined by Western blotting with antibodies to Brm, Rb, E2F1 and E2F4.

(F) Upper image, a DNA containing E2F consensus enhances interaction of C/EBPα with Rb. Nuclear extracts from young livers were incubated with GST-C/EBPα in the presence of short DNA oligomers containing C/EBPα binding site (bZIP) or E2F binding site (DHFR and Ad E2). Rb was determined in GST pull-down samples by Western blotting. Bottom image, a hypothetical model for the C/EBPα-Rb-E2F4-Brm complex. Black box shows an E2F-dependent promoter.

ine whether the age-specific C/EBPα complex interacts with DNA containing C/EBPα or E2F consensuses. Although the gel shift with E2F probes detects the C/EBPα-Rb-E2F4 complex, this complex is not stable under conditions of gel-shift assay and migrated very close to the top of the gel (data not shown). Therefore, we applied an HPLC-based approach to allow separation of high MW complexes. As a preliminary test, we examined whether DNA containing C/EBPα or E2F consensus sequences increases the interaction of C/EBPα with other components of the complex. Short oligomers containing C/EBPα (bZIP) and E2F consensuses for DHFR and Ad E2 promoters were incubated with samples containing nuclear proteins from young liver and GST-C/EBPα. The amounts of Rb in the complexes with C/EBPα are 3- to 3.5-fold increased after incubation of proteins with wild-type E2F consensuses, but not with the bZIP oligomer (Figure 3F). These data indicate that the presence of a DNA containing E2F consensus increases the interaction of C/EBPα with Rb and presumably the formation of C/EBPα-Rb-E2F4-Brm complex.

To further examine if the C/EBPα-Rb-E2F4-Brm complex interacts with the E2F consensus, nuclear extracts from young livers were incubated with wild-type or mutant E2F oligomers and proteins were fractionated by gel filtration. The incubation of nuclear extracts with wild-type E2F consensus leads to a shift of all components of the C/EBPα-Rb-E2F4-Brm complex to higher

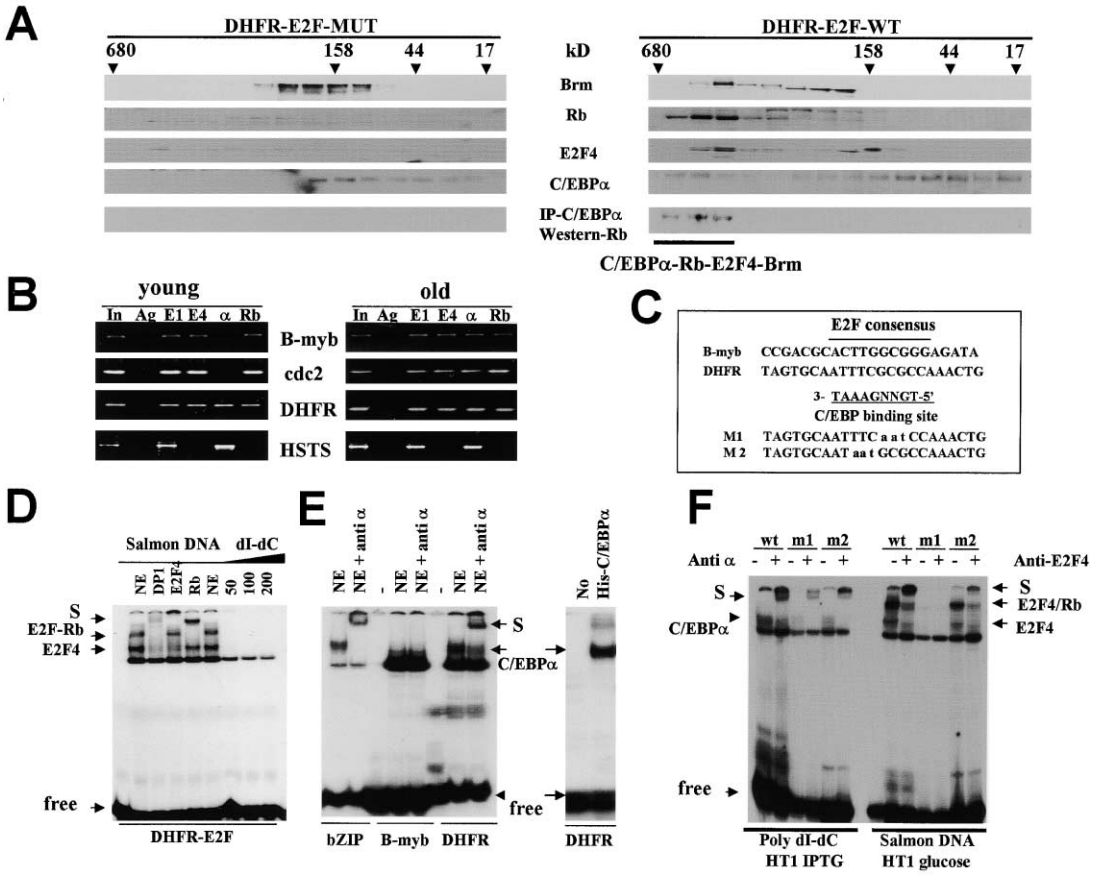

Figure 4. C/EBPα-Rb-E2F4 Complex Binds to E2F Consensus and Occupies E2F-Dependent Promoters in Livers of Old Animals

(A) The presence of a DNA containing E2F consensus increases the formation of C/EBPα-Rb-E2F4-Brm. Nuclear extracts from young livers were incubated with DNA oligomers containing wild-type E2F or mutant E2F consensus, and fractionated by gel filtration. Location of C/EBPα, Rb, Brm, and E2F4 within gel-filtration fractions was determined by Western blotting. Bottom image shows immunoprecipitation of C/EBPα from each fraction and Western blotting with antibodies to Rb.

(B) Chromatin immunoprecipitation (Chip) assay for E2F-dependent promoters; b-myb, cdc2 and DHFR in livers of young and old mice. HSTS, *hydroxysteroid sulfotransferase* promoter (Wells et al., 2002). In, input; Ag, mock control with protein A-agarose only; E1-IP with antibodies to E2F1; E4–IP with antibodies to E2F4; α-IP with antibodies to C/EBPα; Rb-IP with Abs to Rb.

(C) E2F consensus within the DHFR promoter contains a putative site for C/EBPα that overlaps with E2F consensus. Upper image shows a comparison of nucleotide sequences of E2F consensuses within DHFR and b-myb promoters. Bottom indicates mutations (small letters) incorporated into M1 and M2 oligomers. Putative C/EBPα binding site is shown on the top.

(D) Poly dI:dC DNA competitor specifically inhibits E2F binding. Nuclear extracts from HT1 cells were incubated with the DHFR-E2F consensus in binding reactions containing salmon DNA (0.5 μg/reaction) or increasing amounts of poly dI-dC (nanograms, shown on the top). Antibodies to DP1, E2F4 and Rb (shown on the top) were added to the reactions with salmon DNA.

(E) C/EBPα binds to E2F consensus within the DHFR promoter. Left image, nuclear extracts from HT1 cells containing high levels of C/EBPα (see Figure 6A) were incubated with bZIP probe (high affinity site for C/EBPα), b-myb and DHFR-E2F probes in the binding reactions with poly dI-dC competitor. Antibodies to C/EBPα were added to the reactions before probe addition. Right image, bacterially expressed, purified C/EBPα binds to the DHFR-E2F promoter. Electrophoretically homogenous his-C/EBPα was incubated with the DHFR-E2F probe and examined by gel-shift assay.

(F) Mutations that abolish E2F binding also eliminate the interaction of C/EBPα with the DHFR-E2F consensus. Gel-shift assay was performed with WT, M1, and M2 E2F probes (Figure 5C) under conditions optimal for C/EBPα (left) or for E2F (right) bindings. Antibodies to C/EBPα or E2F4 were incorporated in the reactions.

MW fractions, while the incubation of the same extracts with the mutant E2F oligomer (which does not interact with E2F) does not affect the location of these proteins (Figure 4A). The shift of C/EBPα and Rb to high MW fractions is due to the formation of the complex, since Rb is observed in C/EBPα immunoprecipitates from these fractions (Figure 4A). These data indicate that the presence of a DNA containing the E2F consensus is sufficient to promote the formation of the complex and that the C/EBPα-Rb-E2F4-Brm complex interacts with E2F consensuses.

We next examined whether the C/EBPα-Rb-E2F4-Brm complex occupies E2F promoters in livers of old animals. Chromatin immunoprecipitation assays were utilized for these studies. Three E2F-dependent promoters, DHFR, b-myb and cdc2, were initially examined. As an additional control, we performed PCR reactions with primers specific to the hydroxysteroid sulfonyltransferase (HSTS) promoter. The HSTS promoter interacts in vivo with both E2F1 (through non-E2F specific site) and with C/EBPα, but not with E2F4 or with other members of E2F family (Wells et al., 2002). In young animals, b-myb

and cdc2 promoters are occupied by E2Fs and Rb, while in old livers E2Fs, Rb and C/EBPα are located on these promoters (Figure 4B). The difference in the binding of C/EBPα to E2F promoters in young and old animals is specific for E2F dependent promoters, since the control HSTS promoter is bound only to E2F1 and C/EBPα in both age groups. It is interesting to note that although Brm is observed within the C/EBPα complexes in nuclear extracts of old animals, we could not detect in vivo association of Brm with any of the E2F-dependent promoters analyzed in this study, using several sources of antibodies to Brm. Another interesting observation is that, although young animals do not contain detectable levels of C/EBPα-Rb-E2F4 complexes, C/EBPα is located on the DHFR promoter, but not on two other E2F-dependent promoters, cdc2 and b-myb. We further showed that the association of C/EBPα with DHFR promoter in young animals might be due to a direct interaction of C/EBPα with the DHFR promoter (see below). In summary, chromatin precipitation analysis revealed that the C/EBPα-Rb-E2F4 complex is bound to E2F promoters in quiescent livers of old animals.

C/EBPα Directly Interacts with E2F Consensus of the DHFR Promoter

Given the occupation of the DHFR promoter by C/EBPα in young livers, we next examined mechanisms responsible for localization of C/EBPα on the DHFR promoter. Nucleotide sequences of E2F consensuses within the DHFR and b-myb promoters are shown in Figure 4C. A putative C/EBPα consensus (TCGCGAAAT) was found within E2F binding site of the DHFR promoter, but not within b-myb promoter. We next established conditions of gel-shift assay under which E2F binding does not interfere with binding of C/EBPα. It has been previously shown that poly dI-dC competitor specifically inhibits E2F binding (Jansen-Durr, 1994), but does not affect the interaction of C/EBPα with DNA (Timchenko et al., 1996). Figure 4D shows a typical picture of the binding of E2F to the DHFR promoter in reactions containing salmon DNA or poly dI-dC competitors. Both free E2F and E2F-Rb complexes are detectable in the reactions with salmon DNA. However, poly dI-dC completely blocks the interaction of E2F with DNA. Therefore, dI-dC competitor was further used in experiments to monitor only C/EBPα binding activity in extracts containing both E2F and C/EBPα transcription factors. As a positive control for C/EBP binding, a bZIP probe containing high affinity C/EBPα site (Timchenko et al., 1996) was used. Figure 4E shows that C/EBPα binds to the DHFR promoter, but not to b-myb promoter. Incorporation of specific antibodies to C/EBPα supershifted the complex and revealed that C/EBPα interacts with the DHFR promoter. This interaction seems to be direct, because bacterially expressed, purified to homogeneity his-C/EBPα also binds to the DHFR promoter (Figure 4E, right). The mutations of TCGC region within E2F consensus of the DHFR promoter (M1, Figure 4C) abolish binding of both E2F and C/EBPα (Figure 4F). Based on these data, we conclude that C/EBPα directly binds to E2F consensus within the DHFR promoter and that binding sites for E2F and C/EBPα overlap within the DHFR promoter. These data suggest that the presence of C/EBPα on the DHFR promoter in young animals is mediated by the direct interaction of C/EBPα with the DHFR promoter.

C/EBPα-Rb-E2F4 Complex Represses Expression of c-*myc* after PH, which Leads to the Loss of Proliferative Response in Old Animals

Given the occupation of E2F-dependent promoters by the C/EBPα-Rb-E2F4 complex in quiescent livers of old animals, we tested whether this complex represses E2F-dependent transcription after PH. Western analysis of C/EBPα in livers of young and old animals showed that C/EBPα is reduced after PH in livers of young animals, but C/EBPα levels are not altered after PH in livers of old animals (Figures 5A and 5B). We next examined the expression of E2F targets in young and old livers. We chose a short (6 hr for rat liver and 8 hr for mouse liver) time period after PH, since C/EBPα levels are dramatically reduced in young livers at early time points and the effects of C/EBPα reduction most likely to be direct within this time frame. Western blotting showed that cdc2, b-myb, and DHFR proteins are not changed within 6–8 hr after PH in both age groups (Figures 5A and 5B; for DHFR and cdc2, data not shown). On the contrary, c-*myc* protein is dramatically induced after PH in livers of young animals, while no induction of c-*myc* was observed in old animals. These studies demonstrated that the induction of c-*myc* is blocked in old livers of both mice and rats. We examined the interaction of C/EBPα with the c-*myc* promoter and found that C/EBPα does not bind directly to the c-*myc* promoter (data not shown). This suggests that effects on this promoter by C/EBPα may occur through the interaction with Rb and E2F proteins.

We next examined C/EBPα-Rb-E2F4-Brm complex in young and old livers after PH. Analysis of protein levels of Rb, E2F4, and Brm after PH is shown in Figures 5A and 5C. Protein levels of Rb, Brm, and E2F4 are not significantly changed in response to PH in both age groups. At the same time, basal levels of Brm and C/EBPα are very high in livers of old animals after PH. Coimmunoprecipitation assay showed that the C/EBPα-Rb-E2F4-Brm complex is abundant in old livers after PH (Figure 5D). We also detected the C/EBPα-Rb-E2F4-Brm complex by gel-filtration chromatography (data not shown). We next examined whether the failure of old mice to induce c-*myc* is due to the repression of the c-*myc* promoter by the C/EBPα-Rb-E2F4 complex. The occupation of the c-*myc* promoter by E2F-Rb and C/EBPα proteins was examined in quiescent livers and in livers after PH using chromatin-IP assay. As can be seen in Figure 5E, E2F4-Rb complexes are present on the c-*myc* promoter in quiescent young livers. In addition to these proteins, C/EBPα also occupies the c-*myc* promoter in quiescent old livers. The induction of c-*myc* after PH in young mice correlates with the removal of Rb from the c-*myc* promoter, while in old livers the c-*myc* promoter is occupied by the C/EBPα-Rb-E2F4 complex. These observations suggest that the age-specific C/EBPα-Rb-E2F4 complex represses transcription of c-*myc* and perhaps other E2F target genes such as cdc2 (Figure 5E). We also examined whether other signal transduction pathways differ in young and old animals after PH. Gel shift and Western analyses did not show differences in the activation of NF-kB, Stat3, Jun B, and C/EBPβ in young versus old mice (Figures 5A and 5B; for Stat3 and NF-kB, data not shown). A working model for the regulation of the c-*myc* promoter in young and old livers after PH is shown in Figure 5F. Our data sug-

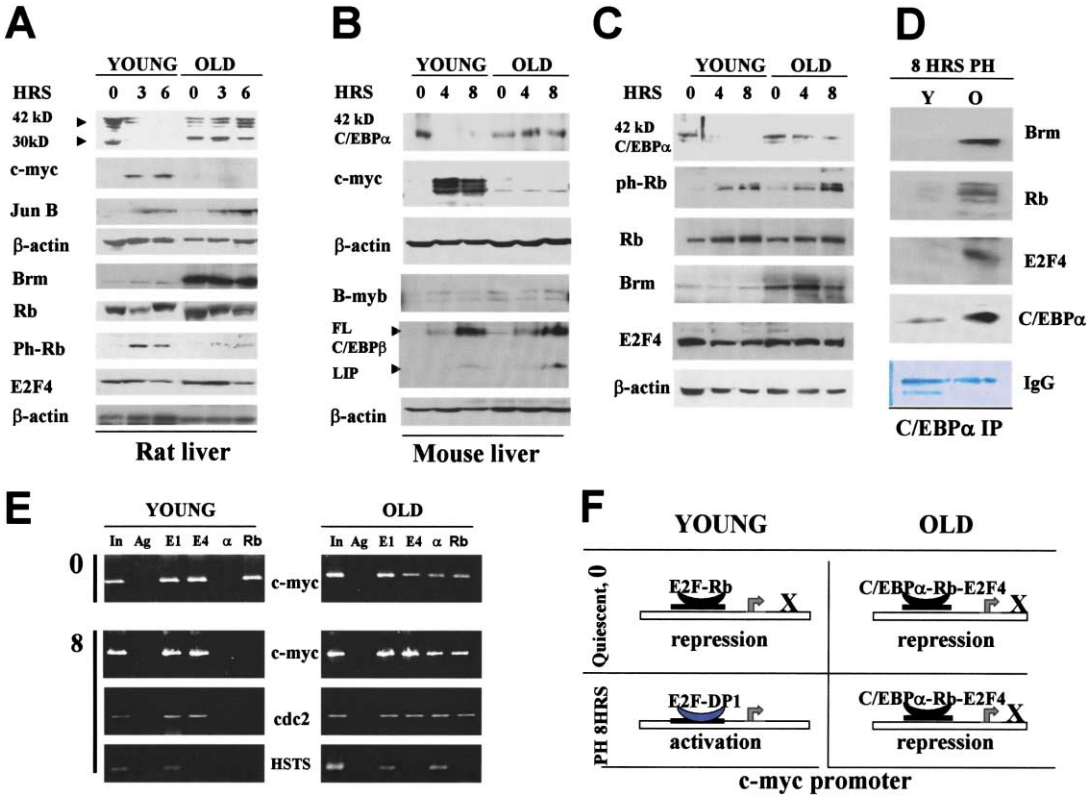

Figure 5. C/EBPα-Rb-E2F4 Complex Represses the c-*myc* Promoter after Partial Hepatectomy in Livers of Old Animals

(A) A failure of old rat livers to reduce C/EBPα after PH correlates with the lack of c-*myc* induction. Proteins extracts from livers of young and old rats were isolated at different time points after PH (indicated on the top), and examined for the expression of C/EBPα, c-*myc*, Jun B, Brm, Rb, and E2F4. Protein loading was verified by reprobing the membranes with β actin.

(B) Old mouse livers also fail to reduce protein levels of C/EBPα and do not induce c-*myc* in response to PH. Expression of the proteins (shown on the left) was examined in young and old mouse livers after partial hepatectomy by Western blotting. Protein loading was verified by reprobing the membranes with β actin.

(C) The components of the C/EBPα-Rb-E2F4-Brm complex are abundant in old mouse livers after PH. Western blotting was performed with antibodies shown on the left as described above.

(D) Old livers contain the C/EBPα-Rb-E2F4-Brm complex after PH. C/EBPα immunoprecipitates from 8 hr PH young and old livers were probed with antibodies shown on the right. IgG, Coomassie blue staining of the membrane after Western blotting. A section with heavy chain IgG is shown.

(E) C/EBPα-Rb-E2F4 complex occupies c-*myc* promoter in liver of old mice after PH. Chromatin immunoprecipitation assay was performed with quiescent livers and with livers 8 hr after PH for young and old animals.

(F) A hypothetical model for the regulation of the c-*myc* promoter after partial hepatectomy in young and old livers (see text).

gest that the loss of c-*myc* induction in old livers is due the repression of this promoter by C/EBPα-Rb-E2F4 complex. In young animals, PH causes phosphorylation of Rb. Following removal of Rb from E2F promoters, activation of c-*myc* and other E2F target genes normally takes place. In old animals, however, the C/EBPα-Rb-E2F4 complex is present on the c-*myc* promoter and blocks its activation.

C/EBPα Represses c-*myc* Promoter through C/EBPα-Rb-E2F4 Complex

Given the lack of induction of the c-*myc* in old animals and the presence of C/EBPα-Rb-E2F4 complex on c-*myc* promoter, we further examined the role of the complex in the repression of c-*myc* expression. To obtain direct evidence that C/EBPα represses the c-*myc* promoter through the C/EBPα-Rb-E2F4 complex, we performed an analysis of C/EBPα-dependent repression of c-*myc* in two stable clonal lines: Rb-positive HT1 clone and Rb-negative SA12 clone. In both cell lines, C/EBPα is placed under Lac-repressor control and can be induced by IPTG. We have previously shown that IPTG-mediated induction of C/EBPα in Rb-positive HT1 cells causes growth arrest (Timchenko et al., 1996). Figure 6 shows the levels of C/EBPα, and the E2F targets DHFR, c-*myc*, cdc2, and b-myb after IPTG addition. C/EBPα levels are increased at 2–24 hr after IPTG addition with the maximum induction at 24 hr. Calculations of DHFR, c-*myc*, cdc2, and b-myb proteins as ratios to β actin show that C/EBPα inhibits expression of all E2F targets including c-*myc*. Chromatin immunoprecipitation assay shows that the C/EBPα-Rb-E2F4 complex occupies the c-*myc* promoter at 24 hr after C/EBPα induction (Figure 6B).

To examine whether the C/EBPα-Rb-E2F4 complex is required for the C/EBPα-dependent repression of c-*myc*, we utilized the stable clonal line (SA12) derived

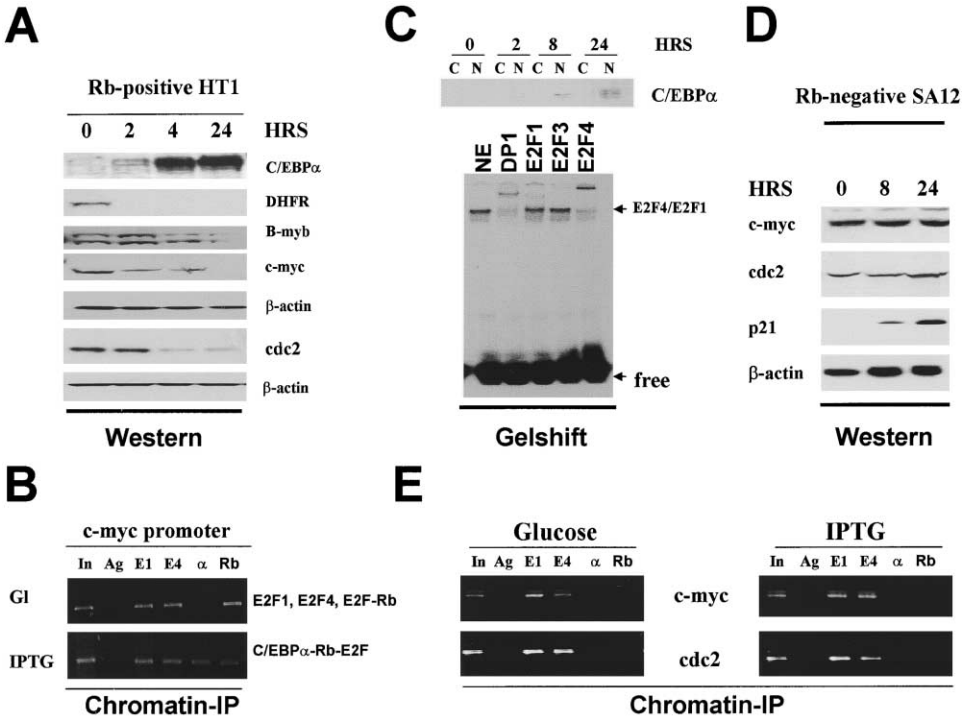

Figure 6. C/EBPα Represses the c-*myc* Promoter through the C/EBPα-Rb-E2F4 Complex

(A) Inhibition of E2F target genes by C/EBPα in Rb-positive HT1 clone. C/EBPα was induced by addition of IPTG and the expression of DHFR, b-myb, c-*myc*, and cdc2 was examined at different time points (indicated on the top) after IPTG addition. Upper image shows levels of C/EBPα. Each membrane was reprobed with β actin to verify protein loading.

(B) C/EBPα−Rb-E2F4 complex represses the c-*myc* promoters in Rb-positive HT1 clone. Chip assay was performed as described in Experimental Procedures. Gl, glucose-treated cells. IPTG, cells expressing C/EBPα. The loading is similar to that described in the legend to Figure 4B.

(C) Generation of a stable C/EBPα clone (SA12) in Rb-deficient SAOS2 cells. Upper image shows Western blotting of cytoplasmic (C) and nuclear (N) proteins at different time point after IPTG addition with antibodies to C/EBPα. Bottom image: Rb and Rb-like proteins are not detectable in complexes with E2F. Gel-shift assay with E2F probe was performed with protein extracts from SA12 clone. Antibodies to E2Fs (shown on the top) were added to the binding reactions.

(D) C/EBPα does not repress the c-*myc* promoter in Rb-deficient cells. Expression of E2F targets, c-*myc* and cdc2, and p21 were determined in SA12 cells at different time points after induction of C/EBPα.

(E) Rb is required for the occupation and repression of the c-*myc* promoter by C/EBPα. Chip assay with c-*myc* and cdc2 promoters in glucose-treated (control) and IPTG-treated SA12 cells was performed as described in Experimental Procedures.

from Rb-deficient SAOS2 cells. Figure 6C shows that the addition of IPTG to SA12 cells leads to the induction of protein levels of C/EBPα and to the induction of C/EBPα binding activity (data not shown). We determined E2F complexes, which are operating in SA12 cells, and found that only DP1/E2F1 and DP1/E2F4 are detectable, while Rb and other Rb family proteins do not form detectable complexes with E2F (Figure 6C, gel shift). We next analyzed expression of c-*myc* and cdc2 and the occupation of corresponding promoters by E2F and C/EBPα complexes at very early times after C/EBPα induction (when the effect is likely to be direct). Protein levels of c-*myc* and cdc2 are not reduced within 24 hr after C/EBPα induction (Figure 6D) suggesting that, in Rb-deficient cells, C/EBPα does not directly repress c-*myc*. This result is consistent with the failure of SA12 cells to form C/EBPα-Rb-E2F4 complex (Figure 3). Chromatin immunoprecipitation analysis of the c-*myc* promoter in SA12 cells expressing C/EBPα (IPTG) or in control cells (glucose) shows that, in Rb-deficient cells, C/EBPα is not bound to the c-*myc* promoter. However, in Rb-positive HT1 cells, C/EBPα is located on the c-*myc* promoter and represses c-*myc* expression (Figures 6A and 6B). Taken together, data in Rb-positive HT1 and in Rb-deficient SA12 clones, show that C/EBPα represses c-*myc* through the formation of the C/EBPα-Rb-E2F4 complex.

Discussion

Aging Switches Pathways of C/EBPα Growth Arrest in Liver

In 1964, Bucher et al. discovered that livers of old animals have a weak proliferative response to PH (Bucher et al., 1964). Although this phenomenon was described long ago, the molecular basis for the age-associated loss of proliferative capacities in the liver has not been elucidated. We observed that the weak proliferative response in livers of old animals correlates with a failure to reduce the liver-specific transcription factor C/EBPα that is a strong inhibitor of cell proliferation (Timchenko et al., 1998). The growth inhibitory activity of C/EBPα was discovered in Dr. McKnight's laboratory more than ten years ago (Umek et al., 1991) and has been the

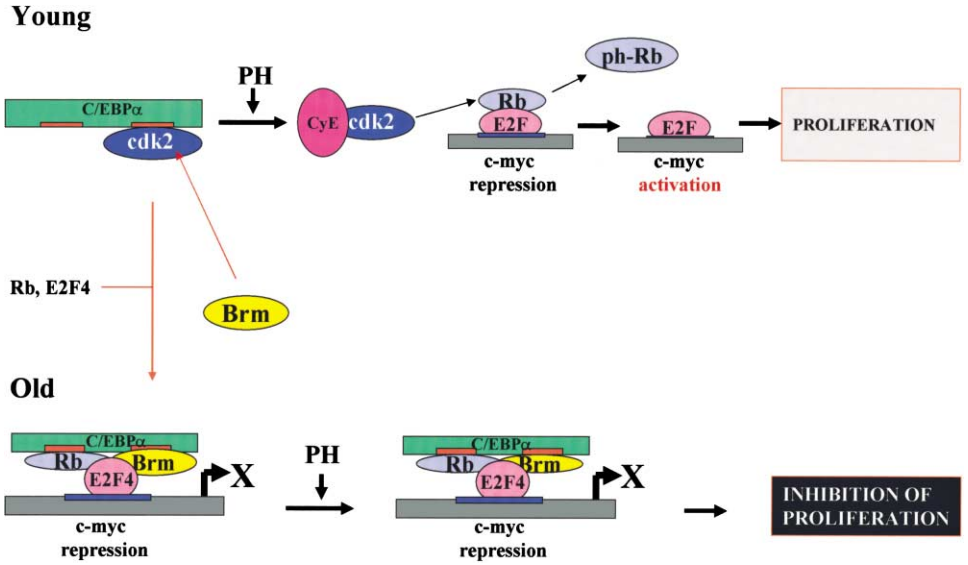

Figure 7. Hypothetical Model for the Loss of Proliferative Response in Livers of Old Animals (see text)

subject of intensive investigations. The major result of these studies is that C/EBPα possesses two major pathways by which it inhibits cell proliferation: direct inhibition of cdks (Wang et al., 2001) and repression of E2F transcription (Porse et al., 2001). These pathways are redundant in cultured cells and are tightly regulated in organisms. A detailed analysis of C/EBPα and interacting proteins in livers of young and old animals showed that, in old livers, C/EBPα is observed in high MW complexes with Rb, E2F4, and Brm. The association of C/EBPα with E2F and Rb family proteins shows that aging switches the C/EBPα growth inhibitory pathway in liver from inhibiting cdks to E2F repression. Our data suggest that this shift is mediated by the induction of Brm levels in old livers. Since Brm and cdks interact with the same region of C/EBPα (Wang et al., 2001; Pedersen et al., 2001), the increased levels of Brm in old livers replace cdks and, together with Rb and E2F4, recruit C/EBPα to the E2F promoters. Our working hypothesis for the failure of old livers to proliferate after PH is presented in Figure 7. Liver proliferation after PH requires an orchestrated cascade of gene expression which includes timed elevation of cyclins D, A, and E. In young animals, cyclins E and A replace C/EBPα from cdk2 and activate cdk2. cdk2 phosphorylates Rb and releases Rb-mediated repression of the c-*myc* promoter. In old animals, however, c-*myc* is repressed by the C/EBPα-Rb-E2F4 complex. This complex is abundant after PH and blocks the induction of the c-*myc* in old livers. A number of investigations demonstrated that the induction of c-*myc* plays a crucial role in liver proliferation after PH (Fausto and Webber, 1994). Arora et al. (2000) recently demonstrated that inhibition of c-*myc* induction after PH in young livers causes a delayed and reduced proliferative response, the situation observed in livers of old animals. Our data suggest that the repression of the c-*myc* by C/EBPα-Rb-E2F4 complex causes the reduced proliferative response in old animals (Figure 7). Old livers are not able to block the E2F pathway of C/EBPα growth arrest, since the elimination of this pathway is not programmed by gene expression after PH.

C/EBPα Represses E2F Transcription as the Component of C/EBPα-Rb-E2F4 Complex

The formation of the C/EBPα-Rb-E2F4-Brm complex requires two regions of C/EBPα: aa 70–95 (Rb interacting region) and aa 140–200 (Brm interacting region). Data from Rb-deficient cells show that Rb is required for the formation of the age-specific complex and for the repression of E2F transcription. The role of Brm in the formation of the age-specific C/EBPα complex is crucial, because the increase of Brm protein levels strongly correlates with the formation of the complex in two biological situations: in old livers and in differentiated adipocytes. Most importantly, the incubation of nuclear proteins from young animals with an excess of Brm leads to the appearance of the complex. These observations demonstrate that Brm is required for the appearance of the C/EBPα-Rb-E2F4-Brm complex. However, the role of Brm in the C/EBPα-mediated repression of E2F targets is not clear at this time. Although Brm is observed in the complexes with C/EBPα, we could not detect Brm on E2F-dependent promoters in vivo. One possible scenario is that Brm is required for the formation of the C/EBPα-Rb-E2F4 complex, and then is removed from the complex after binding to E2F promoters. However, we cannot rule out the possibility that the failure to immunoprecipitate Brm linked to E2F promoters is due to masking of the antibody epitopes after Brm binds to other proteins of the complex. In support of our findings showing the inhibitory role of Brm in liver proliferation, Reyes et al. (1998) demonstrated that $Brm^{-/-}$ livers have an increased rate of liver proliferation.

Several previous publications revealed that C/EBPα inhibits expression of c-*myc* in cultured cells at the transcriptional level (Freytag and Geddes, 1992; Timchenko et al., 1999a; Johansen et al., 2001; Slomiany et al.,

2000). Our data provide the molecular mechanism of this C/EBPα-mediated inhibition: via the repression of the c-*myc* promoter through the formation of C/EBPα-Rb-E2F4 complex. Experiments with two stable clonal lines, HT1 and SA12, revealed that C/EBPα represses c-*myc* promoter in collaboration with Rb and E2F4. It is interesting to note that other E2F targets might be also regulated by C/EBPα through a direct binding to the E2F consensuses. In the course of these studies, we found that C/EBPα binds to DHFR promoter in the absence of the C/EBPα-Rb-E2F4 complex (Figure 4B), and that this binding is mediated by a direct interaction with the E2F consensus within the DHFR promoter (Figures 4C–4E). This finding suggests that C/EBPα might regulate E2F promoters through more than one mechanism.

In summary, data in this paper show that aging changes pathways of C/EBPα growth arrest in liver, and that this change leads to a loss in proliferative response to PH in old animals. The understanding of the molecular basis for the reduced proliferative capacities of old livers is an important step in the development of therapeutic interventions to correct liver proliferation in older organisms.

Experimental Procedures

Animals and Partial Hepatectomy

Fischer 344 rats of 6–8 months (young) and 24 months (old) of age were used in these studies. CB6F1 mice of 4–6 months (young) and 22–24 months (old) were used for the analysis of C/EBPα complexes in quiescent livers and in livers after partial hepatectomy (PH). PH was performed as described in our earlier publications (Timchenko et al., 1998). 70% of the liver was surgically removed and regeneration was allowed to proceed for 4 and 8 hr. Four young and four old animals at each time point after PH were examined.

Gel-Filtration Analysis of C/EBPα Complexes

Nuclear extracts were isolated from livers as described earlier (Timchenko et al., 1997) and fractionated by size-exclusion column SEC-450 (HPLC, BioLogic HR, BioRad). The detailed procedure for the analysis of C/EBPα complexes is described in our previous papers (Wang et al., 2001, 2002). Briefly, gel-filtration fractions were loaded on denaturing gradient (4%–20%) PAAG, blotted onto membrane, and probed with antibodies to C/EBPα (14AA), Rb (C15 or F8), E2F4 (C20 or D3), E2F1, C/EBPβ (C19), Brg1 (from Santa Cruz), or to Brm (Transduction Laboratories). To detect C/EBPα complexes, C/EBPα was immunoprecipitated from each fraction, and IPs were probed with antibodies to Rb or Brm.

Purification of Brm Protein

Brm was purified from nuclear extracts of old livers using a combination of HPLC-based, ion exchange, hydrophobic and size exclusion chromatography columns. The purity and identity of Brm was examined by Coomassie blue staining and by Western blotting with antibodies to Brm. Coomassie blue staining showed that the purified Brm represented a single band with MW 180 kDa that interacted with antibodies to Brm. All preparations were examined for the presence of Rb and E2F4. The purified Brm did not contain detectable amounts of E2F4, Rb, or C/EBPα.

Protein Isolation and Western Blotting

Nuclear extracts were isolated from cultured HT1 or SA12 cells as described in previous papers (Timchenko et al., 1996; Wang et al., 2001).

Western Blotting with Proteins from Stable Clones HT1 and SA12

A stable C/EBPα clone SA12 was generated with Rb-deficient SAOS2 cells as described (Timchenko et al., 1996). C/EBPα was induced by IPTG and proteins were isolated at different time points after C/EBPα induction. Proteins (50 μg) were loaded on gradient (4%–20%) PAAG, transferred on the membrane, and probed with antibodies to C/EBPα and to E2F targets: c-*myc* (N262), b-myb, DHFR, and cdc2 (Santa Cruz). To verify protein loading, each filter was reprobed with β actin and then stained with Coomassie blue.

Western Blotting with Proteins from Quiescent Liver and after Partial Hepatectomy

Nuclear extracts were isolated from mouse livers at 0, 4, and 8 hr after PH, loaded on the gradient (4%–20%) PAAG, and analyzed by Western blotting as described above.

Gel Shift

Conditions for gel-shift assay and DHFR-E2F probe are described in our earlier paper (Timchenko et al., 1999a).

Coimmunoprecipitation and GST Pull-Down

C/EBPα was immunoprecipitated from nuclear extracts with polyclonal antibodies (14AA, Santa Cruz), and the presence of Rb, Brm, E2F4, cdk4, or cdk2 in C/EBPα IPs was examined by Western blotting with monoclonal antibodies to mentioned proteins. GST pull-down assay was performed as described in our papers (Wang et al., 2001, 2002).

Chromatin Immunoprecipitation

The chromatin immunoprecipitation assay was performed with cultured cells and with liver tissues as described in papers from Farnham's lab (Wells et al., 2000, 2002.). The chromatin solution was sonicated to obtain DNA fragments of 500–1000 bp length. Antibodies against C/EBPα (14AA), E2F1 (sc-193), E2F4 (sc-866), Rb (C15) (purchased from Santa Cruz), and Brm (Transduction Laboratories) were added to each aliquot of chromatin and incubated for overnight. Blocked protein-A agarose (Sigma) was added and incubated for 30–40 min at room temperature. After removing the crosslinking proteins, DNA was precipitated and used for PCR reaction with primers specific for E2F-dependent promoters. We used primers to E2F-dependent promoters, which have been characterized in the Wells et al. paper (2000). The sequences of the primers are as follows: DHFR +1360, 5′-TCAGGACTCAGGCTGCTCGAGCCGC-3′; DHFR +962, 5′-CGGCAATCCTAGGGTGAAGGCCTGGT-3′; B-myb +446, 5′-CAGAGCCAGGG CCTCGCCTCATTG-3′; B-myb + 858, 5′-TCAGGACTCAGGCTGCTCGAGCCGC-3′; Cdc2A-20, 5′-GGTAAAG CTCCCGGGATCCGCCAAT-3′; Cdc2B-358, 5′-GTGGACT GTCAC TTTGGTGGCTGGC-3′; C-mycA, 5′-CTTTATAATGCGAGGGTCTG GAGC-3′; and C-mycB, 5′-GCTATGGGCAAAGTTTCGTGGATG-3′. PCR mixtures were amplified for 1 cycle of 95°C for 5 min, annealing temperature for primers for 5 min, and 72°C for 2 min. Then PCR mixtures were amplified for 34 cycles of 95°C for 1 min, annealing temperature for 2 min, and 72°C for 1.5 min. PCR products were separated by 1.5% agarose gel electrophoresis. Three animals of each age group were examined by Chip assay. At least three Chip assays were performed with each individual animal.

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