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Regenerating livers of old rats contain high levels of C/EBPα that correlate with altered expression of cell cycle associated proteins

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ABSTRACT

The nuclear transcription factor, CCAAT/enhancer binding protein α (C/EBPα) is expressed at high levels in the liver and inhibits growth in cultured cells. We have tested the correlation between C/EBPα levels, cell cycle proteins and hepatocyte proliferation in old and young animals as an *in vivo* model system in which the proliferative response to partial hepatectomy (PH) has been shown to be reduced and delayed in old animals. Here we present evidence that the expression of C/EBPα in old rats (24 months) differs from its expression in young animals (6–10 months) during liver regeneration. Induction of proliferating cell nuclear antigen (PCNA), a marker of DNA synthesis, occurs at 24 h after PH in young rats but is delayed and reduced in old animals. Induction of the mitotic-specific protein, cdc2 p34, is 3–4-fold less in regenerating liver of old rats than in the liver of young animals, confirming the reduced proliferative response in old animals. In young rats, the normal regenerative response involves a reduction of 3–4-fold in the levels of C/EBPα protein at 3–24 h. In old animals, C/EBPα is not reduced within 24 h after PH, but a decrease of C/EBPα protein levels can be detected at 72 h after PH. Induction of C/EBPβ, another member of the C/EBP family, is delayed in old animals. Changes in the expression of C/EBP proteins are accompanied by alteration of the CDK inhibitor, p21, which is also decreased in young rats after PH, but in old animals remains unchanged. High levels of p21 protein in older animals correlate with the lack of cdk2 activation. We suggest that the failure to reduce the amount of C/EBPα and p21 is a critical event in the dysregulation of hepatocyte proliferation in old animals following PH.

INTRODUCTION

The effect of age on liver regeneration in rats and mice has been the subject of numerous investigations showing that the proliferative response to partial hepatectomy in older rats is reduced and delayed (1,2). The rate of DNA synthesis in young rats is biphasic with peaks at 22–24 and 35–37 h after partial hepatectomy (PH). In old animals, a delayed, single and broad peak of DNA replication initiating at 30–36 h was observed. The levels of DNA polymerase α and DNA replication in regenerating livers of aging mice were also reduced and delayed compared to young animals (3). Recently, we demonstrated that C/EBPα is a crucial regulator of liver proliferation: hepatocytes in C/EBPα knockout mice had increased DNA synthesis at birth compared to normal animals and hepatocyte proliferation continued at a high rate (30–40%) relative to control littermates (3%) at day 17 of life (4). The observations of this genetic knockout model and the reported delay in the regenerative response in aged animals prompted our examination of the regulation of C/EBPα expression in livers of old rats. The nuclear transcription factor, C/EBPα, a member of the bZIP family of proteins, is expressed at high levels in differentiated hepatocytes and plays a significant role in adipose tissue differentiation (5–8) and in control of cell proliferation. Overexpression of C/EBPα in cultured mammalian cells leads to growth arrest (5,9–11). Although C/EBPα mediated growth arrest of mammalian cells in culture is well documented, the molecular mechanisms by which this factor inhibits growth are not well understood. One pathway of C/EBPα mediated growth arrest was suggested by experiments with a stably transformed human cell line that conditionally expressed high levels of C/EBPα (10). C/EBPα inhibited growth of these human fibrosarcoma cells through the stabilization and elevation of p21 protein level (10). Recently we have shown that the hepatocytes of newborn C/EBPα$^{-/-}$ mice have several characteristics specific to dividing hepatocytes: increased DNA synthesis, low levels of p21 and high levels of the S phase specific protein, PCNA (4).

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Flodby *et al.* also described an increased frequency of PCNA positive hepatocytes in the liver of C/EBPα deficient mice (12). The p21 protein, an inhibitor of DNA synthesis and cyclin dependent kinases (CDKs), was discovered as an mRNA that was overexpressed in senescent fibroblasts (13). p21 is also known as SDI-1 (senescent derived inhibitor-1) (13), WAF-1 (wild type p53 activated fragment 1) (14) and as CIP-1 (CDK interacting protein 1) (15). Investigation of p21 function, carried out in cultured cell lines, has shown that the general mechanism of p21 action is inhibition of cyclin dependent kinases resulting in growth arrest in the G_1 phase of cell the cycle (16). p21 has also been shown to interact with PCNA, a protein that is involved in DNA synthesis, and to inhibit proliferation through this interaction (17). p21 mediated inhibition of CDK activities is complex and depends on whether the p21/cdk complexes contain one p21 molecule (this complex is active) or two molecules of p21 (kinase activity is not present) (18,19).

Here we present evidence that C/EBPα and p21 expression in hepatocytes in old rats (24 months) following PH is regulated differently from young animals and shows significantly delayed reduction in the levels of these proteins compared to their young (6–10 months) counterparts. The regulation of p21 in regenerating livers is likely to be C/EBPα dependent, because C/EBPα can protect p21 from degradation in nuclear extracts (4). Failure to reduce p21 and C/EBPα in old rats is coincident with alteration in the expression of a number of cell cycle associated proteins. We propose that the failure to lower C/EBPα and p21 protein levels is responsible for the delay in the synthesis of DNA which is observed in old rats relative to young.

MATERIALS AND METHODS

Animals and partial hepatectomy

Fischer 344 rats of 6–10 months (young) and 24 months (old) of age were used in these studies. Approximately 70% of the liver was surgically removed and regeneration was allowed to proceed for 30 min, and 3, 6, 12, 24, 48 and 72 h. Three to four animals at each time point were used for young and old rats. Animals were sacrificed under anesthesia. Livers from untreated animals were used as the point 0 controls. Sham operations were performed in parallel to the hepatectomized animals and served as a control for the stress response.

Protein isolation and western analysis

Liver nuclear extracts (NE) were isolated as follows. Livers were homogenized in buffer A containing 25 mM Tris–HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT. Nuclei were pelleted by centrifugation at 5000 r.p.m. for 10 min and washed with buffer A. Supernatant (cytoplasm) was frozen. High salt extraction of proteins was performed by incubation of nuclei with buffer B [25 mM Tris–HCl, pH 7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA and 25% sucrose] for 30 min on ice. After centrifugation, the supernatant (nuclear extract) was divided onto small fractions and kept at –80°C. Western analysis was carried out as described (10). Briefly, 50–100 μg of nuclear proteins were loaded on 12% polyacrylamide–0.1% SDS gel. A 15% gel was used for low molecular weight proteins. After separation, proteins were transferred onto membranes (NitroBind, Micron Separation, Inc.) using electroblotting. To equalize the protein loading, a preliminary filter was stained with Coomassie blue to verify the measured protein concentration. After detection of specific proteins, each filter was reprobed with antibodies to β-actin (Sigma) or with antibodies to cdk4. The β-actin control, shown for westerns presented in the paper, was used for quantitation of protein expression in young and old rats. The level of the proteins was calculated as the ratio to β-actin. Filters were blocked by 10% dry milk, 2% BSA prepared on TTBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) buffer saline. Incubations with primary and secondary antibodies were carried out according to recommendations for each antibody. 0.5% dry milk was added to TTBS and this solution was used for incubation with antibodies. For analysis of the p21 protein, two different antibodies were used: a monoclonal antiserum, cp36 (gift from W.Harper, S.Elledge and E.Harlow) and a polyclonal antiserum M-143 (Santa Cruz Biotechnology). All antibodies showed similar results. Antibodies to C/EBPα (14AA), C/EBPβ (C-19), PCNA, cdk4 and cdc2 p34 were from Santa Cruz Biotechnology. Immunoreactive proteins were detected using the enhanced chemiluminescent (ECL) protocol (Amersham).

RNA isolation and northern analysis

Total RNA was isolated as described (10). Twenty-five μg of total RNA were loaded on 1% agarose–2.2 M formaldehyde gel, transferred onto a membrane and hybridized with specific probes. Each filter was hybridized sequentially with C/EBPα, C/EBPβ and 18S rRNA specific probes as described (10). Quantitation of northerns was performed using phosphor imaging. The levels of C/EBPα and C/EBPβ mRNAs were normalized to the 18S rRNA control.

Stability of p21 protein in nuclear extracts

p21 stability in nuclear extracts was examined as described in our previous paper (4). Briefly, bacterially expressed p21 protein was incubated with NEs from regenerating livers of young and old rats for 30 min at 37°C and analyzed by western blotting with polyclonal (H-164) or monoclonal (187) antibodies as described (4).

Immunoprecipitation and kinase assay

Cdk2 was precipitated from nuclear extracts (200 μg/500 μl) using polyclonal antibodies (Santa Cruz Biotechnology). Immunoprecipitates were resuspended in 30 μl of kinase buffer: 25 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$ and 5 mM DDT. Five μl of the suspension was incubated with 1 μg histone H1 in the presence of 1 μCi of [γ-^{32}P]ATP for 30 min at 37°C. Samples were loaded on 0.1% SDS–12% polyacrylamide gel, blotted on nitrocellulose filter (BioRad) and exposed with X-ray film. To examine the amounts of cdk2 in IPs, the membrane was probed by western assay with antibodies to cdk2. The data described in this paper represent six repeats with two independent isolations of NEs from two animals per each time point.

Immunostaining with antibodies to PCNA

Tissue was fixed overnight in 10% formalin, paraffin embedded and stained for PCNA using anti-PCNA (Dako) and AEC (Vectastain).

RESULTS

The proliferative response after PH is delayed in old rats

A reduction in C/EBPα expression during liver regeneration in rats has been previously described (20–22). Because the reduction in C/EBPα precedes DNA replication in young animals, it has been speculated that the levels of C/EBPα in the normal liver must be reduced for cell proliferation to occur. The delayed response of older animals to PH provided a model in which we could observe whether C/EBPα changes occurred with the same kinetics as in the young or whether C/EBPα correlated with hepatocyte proliferation. To investigate the effect of aging on C/EBPα in liver, we measured this protein by western blotting in young (6–10 months, three per time point) and old rats (24 months). Sham operated rats served as the control. We initially verified that the proliferative response is reduced and delayed in old animals after PH. In young rats, DNA synthesis is reported to take place between 16 and 26 h with a peak at 22–24 h after PH (1,23). Hepatocyte proliferation in young and old animals in our study was investigated by measuring PCNA expression using immunostaining of tissue sections (Fig. 1A) as well as western blotting (Fig. 1B and C). The number of PCNA-positive hepatocyte nuclei at 24 h after PH clearly increases in young, but not in old animals (Fig. 1A). In young animals, ~65% hepatocytes are PCNA positive at 24 h after PH. In old rats, PCNA positive nuclei (50%) are detectable at 48 h indicating a delayed response to PH. Western analysis of PCNA (Fig. 1B) shows that PCNA levels were increased at 24 h after surgery in young animals, indicating a normal proliferative response. Densitometric analysis of PCNA expression showed that PCNA levels in old rats are decreased at 3 and 6 h and return to control levels at 24 h. A slight induction is observed only at 48 h (Fig. 1C). Thus, both the number of cells entering S phase and the total amount of PCNA are reduced in old rats relative to the young animals after PH.

To confirm the reduced proliferative response in old rats, we examined the level of cdc2 p34 protein as a second measure of the proliferative status of the cells. This kinase plays an important role in the transition of cells through G_2 to M phase (24,25). Cdc2 p34 is not detectable in quiescent hepatocytes from either young or old rats. Within 48 h following PH, a strong induction of cdc2 p34 is observed in young rats, however, in old rats the induction is moderate (Fig. 2). The induction of cdc2 p34 in old rats is 3–4-fold less than in young rats. In addition, inducible cdc2 p34 protein in young rats consists of two bands, whereas cdc2 p34 in old rats migrates as a single protein. The upper immunoreactive band seen in regenerating livers is the phosphorylated form of cdc2 p34 as treatment of proteins with increasing amount of alkaline phosphatase (AP) resulted in a shift of the upper band to the position of the bottom one (Fig. 2, lower panel). This observation shows that expression and phosphorylation of cdc2 p34 differs in young and old rats and reflects the degree of proliferation induced by PH. Thus, examination of PCNA and cdc2 p34 expression showed that the proliferative response in old rats is reduced and delayed. To determine the molecular basis for the delayed response in old rats, we examined the expression of C/EBPα which has been shown to inhibit hepatocyte proliferation (4).

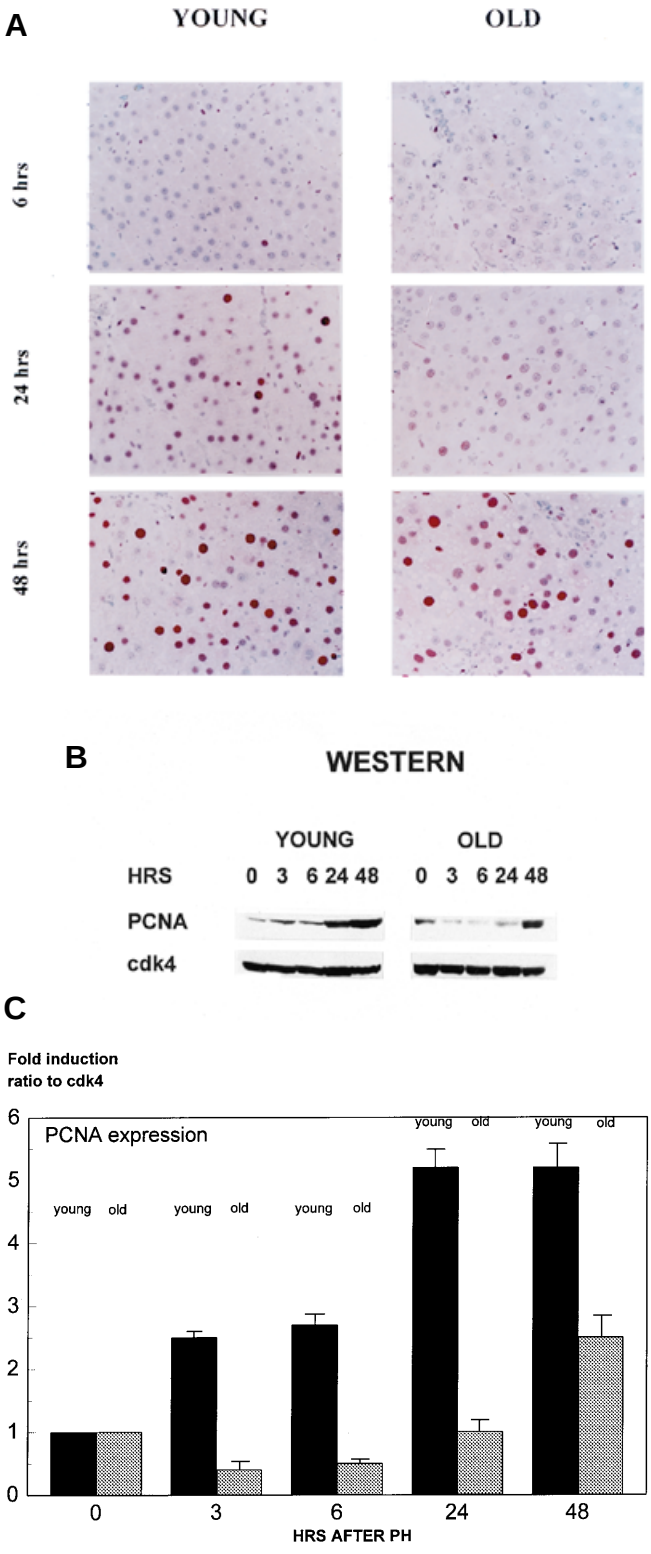

Figure 1. The proliferative response to PH in old rats is delayed. (**A**) Livers from young (6–10 months) and old (24 months) rats were collected at 6, 24 and 48 h after PH (indicated on the left), fixed with formalin and stained with monoclonal antibodies to the S phase specific protein, PCNA. **(B)** Western analysis of PCNA. Nuclear protein (50 µg) isolated at different times after PH (indicated on the top) was analyzed by western analysis with antibodies to PCNA. The membrane was stripped and reprobed with antibody to cdk4. (**C**) Densitometric analysis of PCNA expression. PCNA levels were calculated as a ratio to cdk4. The average of three independent experiments is shown.

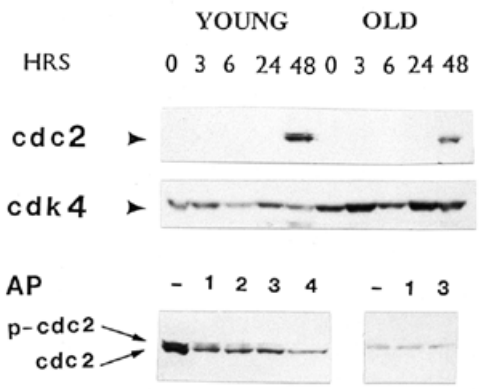

Figure 2. Induction of cdc2 p34 protein in old rats is reduced compared to an induction in young animals. (**Upper**) Western analysis with cdc2 p34 antibodies was performed as described above. Laser densitometry analysis and quantitation of cdc2 p34 levels as the ratio to cdk4 indicated that, in young rats, the level of cdc2 p34 at 48 h is 3–4-fold higher than in old rats. (**Lower**) The upper band of cdc2 p34 is the phosphorylated form of cdc2. Nuclear extracts (NE) from young (left) and old (right) rats were treated with increasing amounts (indicated on the top), alkaline phosphatase (AP). After the treatment, proteins were analyzed by western blotting. Positions of phosphorylated (p-cdc2) and unphosphorylated (cdc2) forms are shown on the left.

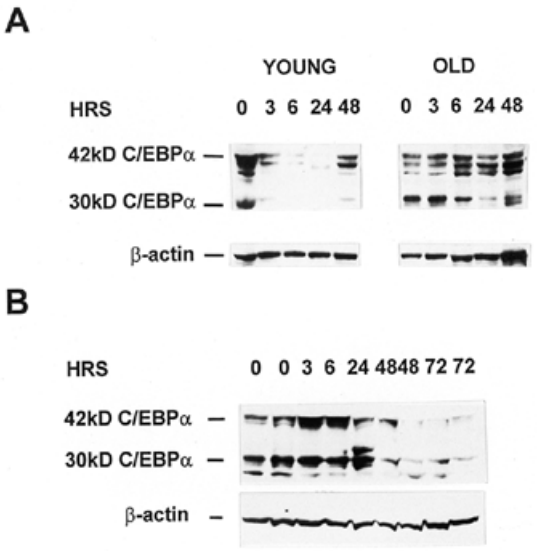

Figure 3. (**A**) C/EBPα protein levels are not reduced in old rats within 24 h in response to PH. Nuclear protein (50 µg) isolated at different times after PH (indicated on the top) were analyzed by western analysis with antibodies to C/EBPα. Western blotting with β-actin was performed after stripping the C/EBPα membrane. (**B**) Reduction of C/EBPα in old animals is shifted to 72 h after PH. Proteins were isolated from old rat livers and analyzed by western with antibodies to C/EBPα. Two animals for 48 and 72 h are shown.

The levels of C/EBPα protein are not reduced in the livers of old rats within 24 h after PH

Reduction of C/EBPα in young animals in response to PH has been described by several groups (20–22). To determine whether the levels of C/EBPα protein were similarly reduced in old animals, we examined C/EBPα protein levels at different times after PH in these animals. Three animals were studied for most time points. Figure 3 shows a representative western analysis. C/EBPα mRNA produces two C/EBPα isoforms, 42 and 30 kDa, presumably by a 'leaky ribosome scanning' mechanism (26–28). A reduction of both C/EBPα isoforms in young rats was observed by 3 h after PH with maximum decrease at 12–24 h, in good agreement with previously published observations (4,20–22). The pattern of C/EBPα expression in old animals is quite different. C/EBPα levels are not reduced. In some old animals, C/EBPα induction was observed at 3 and 6 h (Fig. 3B and data not shown). Sham operated animals did not show changes in C/EBPα expression. There was little variation in C/EBPα expression among the animals at each time point although a slight reduction of C/EBPα was detectable in one old rat at 24 h after PH. Western analysis on nuclear and cytoplasmic proteins showed that C/EBPα is located in nuclei at all stages of liver regeneration in both age groups (data not shown). Thus, the hepatocytes of old rats were shown to maintain C/EBPα levels within first 24 h after surgery, unlike the situation in younger animals. To examine whether older animals never downregulate C/EBPα or whether C/EBPα reduction occurs at a later time, two additional time points were examined: 48 and 72 h after PH. Figure 3B shows western analysis of C/EBPα expression in old rats after PH. C/EBPα protein levels are reduced in old animals between 48 and 72 h after PH. It is interesting to note that PCNA immunostaining in old rats correlates with C/EBPα reduction (Fig. 1). Thus, we conclude that, in older animals, C/EBPα reduction is shifted to a later time. This conclusion is consistent with the observation that although the proliferative response in old animals is reduced, livers finally restore their original mass.

Induction of C/EBPβ in old rats is delayed, but is ultimately greater than in young animals

Expression of another member of the C/EBP family, C/EBPβ, has been shown to be induced in young rats in response to PH. We examined this protein in old animals after PH by northern and western assays. Several published studies showed that, in young rats, expression of both C/EBPβ translation isoforms, LAP and LIP, is induced at 3 and 6 h after PH (21,22). Northern analysis with total RNA from young and old rats isolated at various times after PH showed that, in young rats, induction of C/EBPβ mRNA was consistent with previously published observations. In old rats, however, C/EBPβ mRNA was not induced within this 3–6 h time frame, but was shifted to 12–24 h after surgery (Fig. 4A). Reprobing the filter for C/EBPα shows an inverse pattern of mRNA expression. When C/EBPβ mRNA induction is at its maximum, C/EBPα is reduced. The expression of C/EBPβ protein isoforms in young and old rats is in agreement with the expression of its mRNA. Both C/EBPβ isoforms (LAP and LIP) are induced in old rats at the later times (12–24 h) (Fig. 4B), but to higher levels. Thus, although induction of C/EBPβ protein is delayed in old animals compared to young, the amount of induction is greater. It is interesting to note that the truncated C/EBPβ isoform, LIP, is induced at higher levels in all animals than LAP resulting in a change of the LAP/LIP ratio (Fig. 4B). The relative abundance of C/EBPβ isoforms in young and old rats was ~1:1 suggesting that LIP levels are adequate to inhibit the activity of other bZIP proteins by heterodimerization and may thereby inhibit genes with promoters containing C/EBP consensus sites.

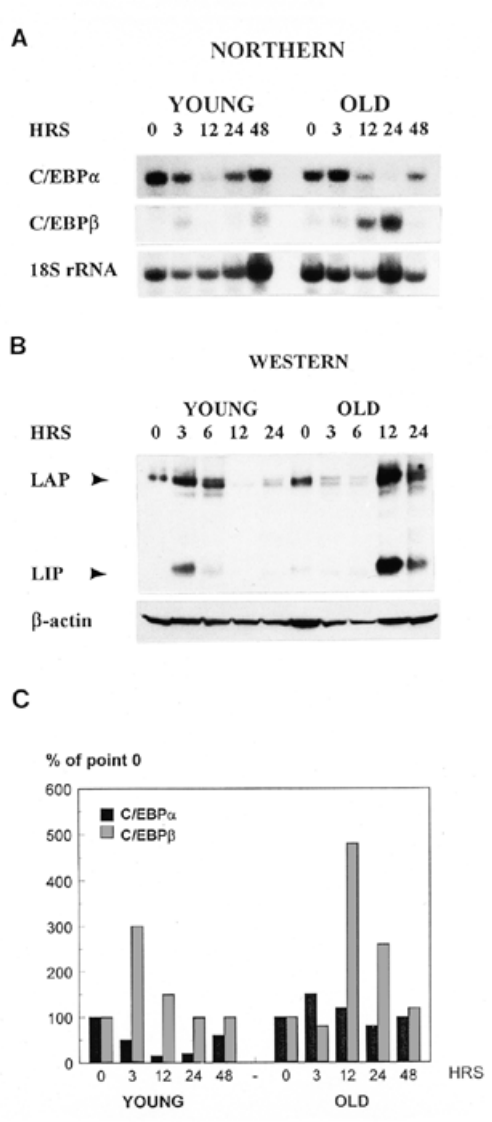

Figure 4. Induction of another member of the C/EBP family, C/EBPβ, occurs slightly later in old rats after PH. (**A**) Northern analysis of C/EBPα and C/EBPβ mRNA expression in young and old rats in response to PH. Total RNA was isolated from rat livers at different times after PH and analyzed by northern with specific probes. The membrane was sequentially hybridized with C/EBPα, C/EBPβ and 18S rRNA probes. (**B**) Induction of C/EBPβ isoforms, LAP and LIP, in old rats is delayed after PH. Western analysis of nuclear extracts from young and old rats was carried out as described above. Positions of the C/EBPβ isoforms, LAP and LIP, are shown on the left. β-actin staining of the same filter shows relative protein loading. (**C**) Quantitation of C/EBPα and C/EBPβ mRNA levels was done using phosphor imaging. The levels of C/EBPα and C/EBPβ mRNA were calculated as the ratio to 18S rRNA. Three independent experiments were used for these calculations.

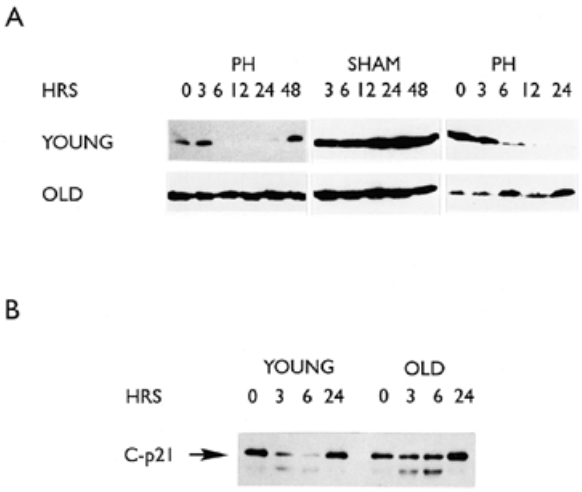

Figure 5. (**A**) Regenerating livers in old animals have high levels of p21 protein. Nuclear proteins (100 μg) isolated from young and old rats at different time points after PH were analyzed by western blotting with p21 specific antibodies. Analysis of two sets of hepatectomized animals is shown. Sham operated rats served as the control. (**B**) Stability of bacterially expressed C-p21 in nuclear extracts isolated from young and old animals at different time points (indicated on the top) after PH. C-p21 was incubated with 5 μg of NE for 30 min as described in Materials and Methods and analyzed by western with polyclonal antibodies H-164 (SC).

Alterations in p21 protein levels correlate with C/EBPα levels

C/EBPα is an inhibitor of proliferation in cultured cells and in developing liver and regulates the level of p21 protein (4,10). The reduction of these two proteins at 6–24 h frame after PH may be necessary to allow normal proliferation of hepatocytes (4). We speculated that the expression of p21 protein might be changed in old rats because of the alterations in the response of C/EBPα to PH. Figure 5A shows a representative western with two sets of young and old rats. Sham operated animals were used as the control and these did not show changes in p21 expression after surgery. The p21 level in young rats was dramatically reduced at 3–24 h, however, in old animals no reduction was observed. Some old animals showed a slight induction of p21 at different times after PH (Fig. 5A). Thus, in hepatocytes of old rats, p21 protein does not decline in response to PH.

p21 degradation is reduced in liver nuclear extracts from old rats

We have previously found that p21 protein is the subject of specific degradation in nuclear extracts isolated from livers of young animals at 3 and 6 h after PH (4). We have also shown that bacterially expressed C/EBPα can protect p21 degradation in these extracts (4). Since older animals do not reduce C/EBPα levels at 3 and 6 h, one possible mechanism of p21 stabilization is that C/EBPα blocks its degradation. Therefore, we examined the stability of bacterially expressed, purified p21 in nuclear extracts from young and old rats. To distinguish bacterially expressed p21 from the endogenous protein, a C-terminal portion of p21 (C-p21) was used in these experiments. Figure 5B shows that degradation of C-p21 in older animals is significantly reduced compared to that in nuclear extracts from young rats (containing lower levels of C/EBPα) at 3 and 6 h post-hepatectomy. This observation is consistent with a C/EBPα mediated stabilization of p21 in liver (4) and suggests that the altered regulation of C/EBPα and p21 in old rats after PH, in turn, changes the kinetics cell cycle progression.

C/EBPα down-regulates cdk2 kinase activity in the liver of newborn mice and in older animals after PH

p21 has been shown to interact with cdk2 and control its kinase activity in cultured cells (15). Given the failure of old rats to reduce

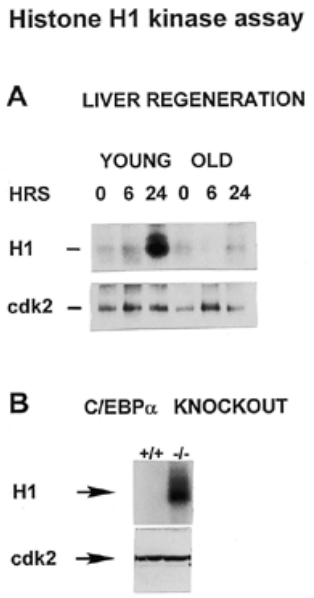

Figure 6. (**A**) Activation of cdk2 does not occur in old animals in response to PH. Cdk2 was precipitated from nuclear extracts isolated at 0, 6 and 24 h after PH. Histone H1 kinase activity of the cdk2 was examined as described in Materials and Methods. The same membrane was probed with antibodies to cdk2. (**B**) Cdk2 kinase activity is induced in the absence of C/EBPα. Cdk2 kinase activity was analyzed in IPs from C/EBPα knockout livers (–/–) and from genetically normal littermate (+/+). The bottom panel shows the level of cdk2 in livers.

C/EBPα and p21 in response to PH, we asked whether cdk2 activity might be altered. To examine this possibility, we determined cdk2 kinase activity in young and old rats in response to PH. Cdk2 was immunoprecipitated from nuclear extracts and its activity was examined using histone H1 as a substrate. Figure 6A shows that cdk2 kinase activity is induced in young animals in 24 h after PH in agreement with previously published observations. However, in old rats, cdk2 kinase activity is not changed. Reprobing the same filter with antibodies to cdk2 showed identical amounts of cdk2 in IPs. We suggest that the kinase activity of cdk 2 is regulated during liver regeneration by p21 protein whose levels are reduced at 24 h in young animals, but not in old rats. To determine whether cdk2 activity is regulated by C/EBPα in liver, we studied cdk2 kinase activity in wild type and in C/EBPα knockout livers. When cdk2 is immunoprecipitated from liver extracts derived from both genotypes and its kinase activity measured by phosphorylation of histone H1, we observed that cdk2 in C/EBPα knockout mice is ~10-fold more active (Fig. 6B). Because p21 levels are reduced in C/EBPα knockout livers, we suggest that this reduction leads to induction of cdk2 activity. This observation shows that, in liver, C/EBPα controls cdk2 kinase activity presumably through the regulation of p21 protein levels.

DISCUSSION

C/EBPα levels decrease before DNA synthesis in regenerating rat livers. Older animals serve as a model to test a hypothesis that a decrease in C/EBPα must take place before hepatocyte proliferation can proceed. If C/EBPα expression has no effect on cell growth *in vivo*, a reduction of C/EBPα might be predicted to occur normally despite the delayed initiation of DNA synthesis. Liver regeneration is affected by the age of the animal: in old animals, the proliferative response to PH was significantly delayed although full restoration of liver mass eventually occurred (1,2). While the molecular basis for this delay is not known, alterations in the expression of numerous proteins have been well documented in regenerating hepatocytes of young animals (23). C/EBP proteins are abundant in liver and are good candidates for regulation of hepatocyte proliferation because of their role in growth arrest of cultured cells (5,9–11). Their growth related activities *in vivo*, however, are not clearly understood. C/EBPα is highly expressed in quiescent hepatocytes and is reduced when hepatocytes undergo division (20–22). Because C/EBPα is an inhibitor of cell proliferation, the high levels of C/EBPα in regenerating livers of old rats might well act to maintain the non-dividing homeostatic condition by affecting the expression of cell cycle associated proteins. Here we examine the kinetics of expression of p21, cdk2 and cdc2 p34 to better understand the molecular changes that account for hepatocyte proliferation. C/EBPα has been shown to elevate the protein levels of p21 in cultured cells (10,29). Our results with newborn and suckling C/EBPα knockout mice showed that, in the absence of C/EBPα, p21 levels are greatly reduced and hepatocyte proliferation is increased at birth and continues for up to 2 weeks, at which time, genetically normal hepatocytes have a low level of proliferation (4). In this paper, we show that regulation of C/EBPα expression in old rats is deranged following the regenerative stimulus of PH. The expression of the cell cycle associated proteins, (PCNA and cdc2 p34), putative targets of the p21 is altered. In addition to the observation that older animals failed to reduce C/EBPα protein levels in response to PH, the C/EBPα mRNA was shown to be 2–3-fold induced in old animals at 3 h after PH. We have observed that, in some old animals, change in C/EBPα mRNA levels does not always lead to expected alterations in protein levels, suggesting that post-translation events are also involved in the regulation of C/EBPα levels in liver. Flodby *et al.* have also described that although the C/EBPα protein reduction correlates with a decrease of mRNA, the kinetics of reduction are slightly different (22). Because expression of C/EBPα mRNA during liver regeneration has been shown to be regulated at the level of transcription (22), we suggest that old animals have lost the appropriate transcriptional control of this gene. A careful analysis of C/EBPα promoter will be required to understand the multitude of regulatory factors that influence C/EBPα gene expression. The proximal region of rat C/EBPα was recently characterized by Rana *et al.* (30) and these *in vitro* studies of HepG2 cells showed that C/EBPβ was a positive regulator of the C/EBPα promoter. We have found that the induction of C/EBPβ is delayed in old rats after PH. In both young and old rats the induction of C/EBPβ correlates with the time of C/EBPα mRNA reduction (Fig. 4) suggesting that C/EBPβ might be a negative regulator of C/EBPα expression *in vivo*.

Five proteins showed significant changes in their levels in old rats compared to young: C/EBPβ, C/EBPα, p21, PCNA and cdc2 p34. The p21 protein has been shown to be reduced in the livers of newborn mice deficient for C/EBPα (4). C/EBPα mediated control of p21 protein in liver does not involve transcriptional regulation of the p21 gene, but rather increases in p21 protein levels. We have shown that C/EBPα interacts with p21 protein in mammalian cells suggesting that this interaction contributes to p21 stability (4). The mechanism of p21 degradation in liver is unknown. We have observed that, in liver nuclear extracts *in vitro*, and in growth arrested HT1 cells, stability of p21 correlates with

high levels of C/EBPα (10) strongly suggesting that this stabilization is a part of the mechanism of C/EBPα mediated growth arrest. Using C/EBPα deficient animals we showed that C/EBPα regulates cdk2 kinase activity presumably through the p21 protein (Fig. 6B). Failure to increase cdk2 activity in old rats correlates with high p21 levels in cdk 2 immunoprecipitates. Our data with C/EBPα knockout livers and with older animals suggest that a p21 dependent pathway of C/EBPα mediated growth arrest in liver involves direct inhibition of cdk2 kinase activity by p21. Progression from G_1 through the cell cycle leads to elevation of cdc2 p34 in young animals, however, cdc2 p34 induction fails to occur with the same kinetics in old rats. In the present study, we have also detected alterations in the expression of two other genes that are required at G_2/M, PCNA and cdc2 p34. Experiments with cultured cells have shown that PCNA interacts with p21 (16,17) leading to the speculation that alterations of PCNA expression in old rats are dictated by high levels of C/EBPα and/or p21. Cdc2 p34 is a mitotic specific protein that is necessary for transition through the G_2/M phase (24,25). Its expression in quiescent rat livers is undetectable. At 48 h, the cdc2 p34 level is induced in both young and old rats, but the level of induction is 3–4-fold less in old rats compared to young. In addition, the phosphorylation of cdc2 p34 does not occur in old rats as it does in young animals. The levels of these two cell cycle related proteins, PCNA and cdc2 p34, are altered in old rats as would be predicted by the smaller fraction of cells entering the cell cycle. It is also possible that migration of cdc2 p34 to the nucleus is affected in older animals as nuclear extracts were used for these studies. *In vitro* experiments with cultured HT1 cells growth inhibited by C/EBPα expression also showed that cdc2 p34 was depressed (data not shown). Thus, our results demonstrate multiple changes in the expression of the genes that are necessary for G_1/S transition in old animals compared to young.

The many alterations observed in cell cycle associated proteins would suggest that one or more pathways of the hepatocyte regenerative response are active in young, but not old animals. Clearly C/EBPα and p21 are expressed in different ways following PH in the two age groups studied and their continued high level of expression is consistent with a delay and reduction in the regenerative response. Other pathways must operate in older animals to 'push' the hepatocytes into cell division, because eventually sufficient numbers of cells undergo replication to replace liver mass. Examination at 72 h suggests that events upstream, or that control and precede changes in the C/EBPα gene, ultimately take place in the old animals. However, our data show that the proliferative response did not take place in the absence of a reduction in C/EBPα.

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