Oncogene (1998) 16, 2141–2150

http://www.stockton-press.co.uk/onc

Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver

Jeffrey H Albrecht[1,3], Randy YC Poon[4], Cory L Ahonen[3], Brenda M Rieland[3], Chuxia Deng[5] and Gretchen S Crary[2]

Departments of [1]Medicine and [2]Pathology, Hennepin County Medical Center, Minneapolis, Minnesota 55415; [3]Minneapolis Medical Research Foundation, Minneapolis, Minnesota 55404, USA; [4]Department of Biochemistry, Hong Kong University of Science and Technology, Kowloon, Hong Kong; [5]Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

In tissue culture systems, p21 and p27 inhibit cyclin-dependent kinase (CDK) activity and cell cycle progression in response to numerous stimuli, but little is known about their involvement in cell growth *in vivo*. We examined the modulation of CDK activity by these proteins after 70% partial hepatectomy (PH), an *in vivo* model of synchronous hepatocyte cell cycle progression. After PH in BALB/c mice, p21 was induced during the prereplicative (G1) phase and was maximally expressed after peak hepatocyte DNA synthesis. p27 was present in quiescent liver and was minimally induced after PH. p21 and p27 immunoprecipitated with CDK2, CDK4, and cyclin D1 in the regenerating liver. The activity of CDK2-, CDK4- and cyclin D1-associated kinases was upregulated after PH, and maximal activity of these enzyme complexes corresponded to peak DNA synthesis. Immunodepletion experiments suggested that p27 plays a role in downregulating CDK2 activity before and after peak DNA synthesis. Compared to cogenic wild-type mice, p21−/− mice demonstrated evidence of markedly accelerated hepatocyte progression through G1 phase after PH: DNA synthesis, upregulation of cyclin A and PCNA, induction of cyclin D1- and CDK2-associated kinase activity, and appearance of a phosphorylated retinoblastoma protein (Rb) species occurred earlier in the p21−/− mice. These results suggest that p21 and p27 modulate CDK activity in the regenerating liver, and that p21 regulates the rate of progression through G1 phase of the cell cycle *in vivo*.

Keywords: cyclins; cyclin-dependent kinases; liver regeneration; G1 phase

Introduction

Compensatory hyperplasia of the liver (liver regeneration) is an example of precisely controlled cell proliferation *in vivo*. In the adult, hepatocytes are highly differentiated, yet they retain a remarkable ability to proliferate in response to acute hepatic injury. In the most extensively studied model of liver regeneration, that of 70% partial hepatectomy (PH) in rodents, most of the remaining parenchymal hepatocytes rapidly enter the cell cycle, and liver mass is restored within 1–2 weeks (Steer, 1995). During the first 1–2 days of this process, a large population of hepatocytes progresses through the cell cycle in a relatively synchronous manner; PH is therefore a useful *in vivo* model of cell cycle progression. The extracellular factors and intracellular events that induce hepatocyte proliferation in the regenerating liver are incompletely characterized but are an area of active interest (Fausto *et al*., 1995). Furthermore, little is known about the mechanisms that inhibit proliferation and return hepatocytes to quiescence after regeneration is complete (Michalopoulos and DeFrances, 1997).

Progression through the cell cycle is controlled by the activity of cyclin-dependent kinases (CDKs), which in turn is regulated by cyclins, CDK-inhibitory proteins (CKIs), and changes in the phosphorylation status of the CDKs (Morgan, 1995; Grana and Reddy, 1995). Activation of CDK4 is thought to play a critical role in progression through the G1 restriction point, when the cell becomes committed to proceed through the cell cycle (Pardee, 1989; Sherr, 1996). CDK2 activation plays an essential role in the transition into S phase and DNA synthesis (Pines, 1995). The activity of these kinases is influenced by numerous mitogenic and antiproliferative signals. The best characterized cyclin partner of CDK4 is cyclin D1; this protein is induced during progression though G1 phase, which is thought to be a key growth factor-dependent step during proliferation (Sherr, 1996). CDK2 is associated with the E- and A-type cyclins during the G1-S transition and S-phase, respectively. In addition to binding to cyclins, full activation of the CDKs requires phosphorylation by the CDK-activating kinase (CAK), which is constitutively expressed in many cells (Sclafani, 1996).

The activity of cyclin/CDK complexes is negatively regulated by the CKIs, which are grouped into two structurally related families (Sherr and Roberts, 1995). The INK4 family (p15, p16, p18, and p19) inhibits CDK4 and CDK6, while the Cip/Kip family (p21, p27, and p57) inhibits numerous CDKs. Of these, p21 and p27 have been the most extensively studied. p21 acts downstream of numerous signaling pathways, and can be induced by p53, transforming growth factor β (TGFβ), and other antimitogenic stimuli (el-Deiry *et al*., 1993; Datto *et al*., 1995; Li *et al*., 1995; Akashi *et al*., 1995). In tissue culture systems, p21 is upregulated in proliferating cells and appears to play a role in governing progression through the cell cycle (Nakanishi *et al*., 1995; Macleod *et al*., 1995; Missero *et al*., 1996). In addition, p21 is induced in some cell types during senescence and terminal differentiation, and it is thought to play a key role in downregulating CDK

Correspondence: JH Albrecht
Present address: Department of Medicine (865B), Hennepin County Medical Center, 701 Park Avenue, Minneapolis, MN 55415, USA
Received 14 October 1997; accepted 18 November 1997

activity in these settings (Noda *et al*., 1994; Guo *et al*., 1995; Marx, 1995). Furthermore, *in vitro* studies suggest that p21 inhibits stress-activated protein kinase (SAPK) activity and proliferating cell nuclear antigen (PCNA)-directed DNA replication (Shim *et al*., 1996; Waga *et al*., 1994; Luo *et al*., 1995). Finally, at low stoichiometric concentrations, p21 may serve as an assembly factor for active cyclin/CDK complexes (Zhang *et al*., 1994; LaBaer *et al*., 1997). However, the importance of p21 in the control of cell proliferation *in vivo* is unclear, since p21 knockout (p21−/−) mice do not exhibit developmental abnormalities, increased tumorigenesis, or hyperproliferative disorders (Deng *et al*., 1995).

p27 is constitutively expressed in many cells, and the level of this protein is elevated in G0 phase and declines as cells in culture enter the cell cycle (Poon *et al*., 1995; Agrawal *et al*., 1996; Polyak *et al*., 1994). p27 may play an important role in governing the growth factor restriction point by ensuring that CDK activity is suppressed during G0 and early G1 phase (Coats *et al*., 1996; Soos *et al*., 1996). In addition, p27 has been implicated as a mediator of growth arrest due to TGFβ, cAMP, and other extracellular factors (Polyak *et al*., 1994; Kato *et al*., 1994). p27 appears to play an important role in regulating cell proliferation during development, since p27 knockout mice demonstrate hyperplasia of numerous organs (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996).

Most studies examining the function of p21 and p27 have been performed in tissue culture systems using transformed cell lines or embryonic cells. Previous studies have suggested that there are unique features of cyclin and CDK regulation in the liver after PH as compared to cells in culture (Lu *et al*., 1992; Loyer *et al*., 1994; Albrecht *et al*., 1995; Kren and Steer, 1996; Castro *et al*., 1994). The purpose of these studies was to examine the potential role of p21 and p27 during hepatocyte growth *in vivo*. The results suggest that p21 and p27 expression differs from that described in other systems, and that these CKIs play distinct roles in the regulation of CDK activity during liver regeneration.

Results

Expression of p21 and p27 proteins during liver regeneration

In the adult liver, hepatocytes are normally quiescent (G0 phase) but can be induced to proliferate in response to injuries that diminish hepatic mass. After PH in male BALB/c mice, a large population of hepatocytes progresses through the cell cycle in a relatively synchronous manner, with peak DNA synthesis occurring 36 h after the procedure (Cornell *et al*., 1990; Albrecht *et al*., 1994). At later time points, DNA synthesis is diminished and represents asynchronous hepatocyte replication and division of nonparenchymal liver cells (Grisham, 1962; Bucher, 1963). By 120 h after the procedure, DNA synthesis returns to near-basal levels. To study the expression of p21 and p27 in this model, we performed standard PH on male BALB/c mice, followed by tissue harvest at time points representing G0 phase (0 h), G1 phase (3, 12 and 24 h), S phase (36 h), and the 'postreplicative' phase of liver regeneration (48–120 h) (Michalopoulos and DeFrances, 1997; Haber *et al*., 1993). At the specified time points, the livers were excised and frozen in liquid nitrogen for later experiments.

To examine the possible role of p21 and p27 protein during liver regeneration, Western blot experiments were performed on homogenates of liver tissue from male BALB/c mice after PH. As is shown in Figure 1a, no p21 was detected in quiescent liver tissue, consistent with the lack of p21 expression noted in normal human liver (el-Deiry *et al*., 1995). The p21 protein was markedly induced after PH, beginning during G1 phase (12–24 h after PH) and peaking during the postreplicative phase (48–72 h). At 120 h after PH, p21 was again barely detectable. In contrast, p27 protein was expressed in quiescent liver and only minimally induced during the regenerative process. As previously shown, the 36 kDa cyclin D1 protein was induced after PH, beginning during G1 phase at 12–24 h and peaking at 72 h, while CDK4 expression varied little after PH (Albrecht *et al*., 1995). Cyclin A protein was induced sharply during S phase (36 h) and its expression declined in the postreplicative phase (48–72 h). Western blot analysis of CDK2 using an antibody directed against the C-terminus of this protein revealed two bands at ~33 kDa that comigrated with bands observed in proliferating Swiss 3T3 cells and human Jurkat cells (data not shown). The faster-migrating

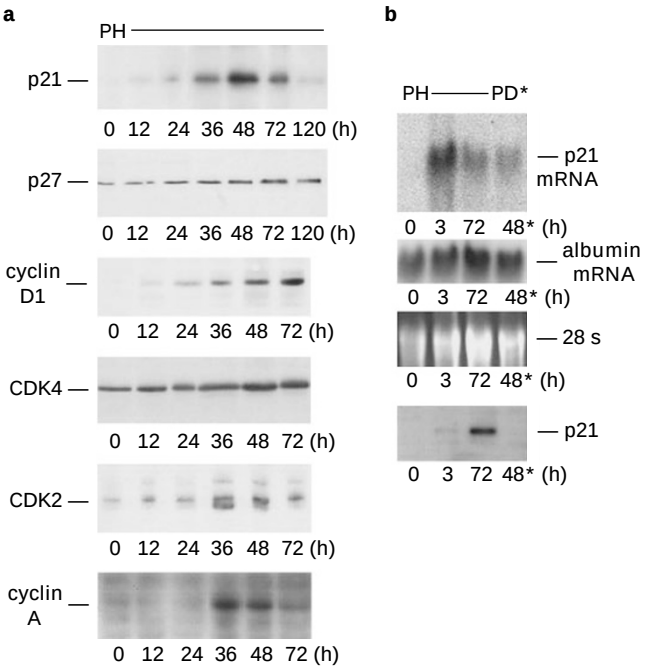

Figure 1 Expression of p21, p27 and cell cycle proteins in regenerating mouse liver. Male BALB/c mice were subjected to 70% partial hepatectomy (PH) followed by harvest of the remnant liver tissue at the indicated time points. (**a**) Western blot analysis. Protein extracts (50 μg/lane) were prepared from the livers and Western analysis was performed with the indicated antibodies, as described in Materials and methods. (**b**) Uncoupling of p21 mRNA and protein expression. Mice underwent PH or protein deprivation (PD*) and the livers harvested as indicated. RNA and protein were isolated from one liver at each time point and used for Northern and Western analysis. The Northern blot (25 μg total RNA/lane) was hybridized with ^{32}P-labeled cDNA probes to p21 and albumin, as described in Materials and methods. The ethidium bromide-stained 28S ribosomal band is also shown. The bottom panel shows Western analysis for p21

form of CDK2 in the regenerating liver, probably representing the Thr160-phosphorylated species (Poon *et al*., 1994), was upregulated 36–48 h after PH. The upper band of CDK2 protein was present in quiescent liver and was less markedly induced during regeneration.

The pattern of p21 protein expression after PH shown in Figure 1a differed substantially from the previously reported expression of p21 mRNA (Albrecht *et al*., 1997). To confirm that p21 mRNA and protein were discordantly regulated, mRNA and protein isolated from one liver at each time point were subjected to Northern and Western analysis, respectively (Figure 1b). As documented previously, p21 mRNA was more markedly induced at 3 h after PH (early G1 phase) than at 72 h. In contrast, p21 protein expression was barely detectable at 3 h but was dramatically upregulated at 72 h. Dietary protein deprivation leads to induction of p21 mRNA in a p53-dependent manner in the liver (Albrecht *et al*., 1997), and results in the expression of other growth-related genes thought to reflect the transition of hepatocytes from G0 to G1 phase (Mead *et al*., 1990). As is shown in Figure 1b, protein deprivation led to the induction of p21 mRNA but not protein, consistent with the concept that this dietary manipulation causes hepatocytes to enter a state resembling early G1 phase.

Interaction of p21 and p27 with cyclin D1, CDK2 and CDK4 after PH

To further examine whether p21 and p27 play a role in the regulation of CDK activity in the regenerating liver, the formation of cyclin/CDK/CKI complexes was evaluated by immunoprecipitation-Western blot studies. Immunoprecipitation with antibody to CDK4 (Figure 2a) demonstrated that cyclin D1/CDK4 complexes became detectable during G1 phase (12–24 h) and paralleled the expression of cyclin D1 protein, consistent with previous results (Albrecht *et al*., 1995). The abundance of cyclin D1/CDK4 complexes peaked at 48–72 h after PH, and then declined at later time points (data not shown).

Immunoprecipitation with antibody to p21 revealed that little cyclin D1, CDK4, or CDK2 associated with p21 in normal (0 h) liver (Figure 2b). After PH, p21 was noted to form complexes with cyclin D1, CDK4, and CDK2 during G1 phase at 12–24 h, and the abundance of these complexes peaked during the postreplicative phase 48–72 h after PH. At later time points (96–120 h), the amount of cyclin D1, CDK4, and CDK2 associated with p21 diminished (data not shown). The formation of p21/cyclin D1 and p21/CDK4 complexes roughly paralleled the induction of p21 and cyclin D1 proteins (Figure 1a), as well as the abundance of cyclin D1/CDK4 complexes. Similarly, p21/CDK2 complexes increased in parallel with the amount of p21 protein in these extracts. These results demonstrate that p21 formed complexes with cyclin D1, CDK2, and CDK4 during the G1, S, and postreplicative phases of liver regeneration after PH.

Although both p27 and CDK4 were present, immunoprecipitation with anti-p27 antibody revealed little detectable p27/CDK4 complexes in quiescent liver (Figure 2c). Similarly, no p27/cyclin D1 complexes were detected in normal liver. After PH, however, formation of p27/cyclin D1 and p27/CDK4 complexes was noted during G1 phase (12–24 h). The amount of these complexes increased only modestly from 24–72 h and did not clearly correlate with the abundance of cyclin D1/CDK4 complexes. p27/CDK2 complexes were present in quiescent liver, and their abundance increased slightly after PH. We were unable to detect p21 and p27 in the same complex after immunoprecipitation with antibodies to either protein (data not shown). p27 did associate with cyclin D1, CDK2, and CDK4 at each time point after PH.

Cyclin D1, CDK2, CDK4, and CAK activity in regenerating liver

After PH in the rat, induction of CDK1- and CDK2-associated kinase activity correlates with progression through S phase (Lu *et al*., 1992; Loyer *et al*., 1994). We examined the induction of cyclin D1-, CDK2- and CDK4-associated kinase activity after PH in male BALB/c mice (Figure 3). ln each case, maximal enzyme activity was noted at 36 h after PH, corresponding to S phase in this model. At 48 h, the activity of each enzyme complex was still elevated, while at 72 h kinase activity returned to near-basal levels. Thus activation of these cyclin/CDK complexes after PH correlated well with their putative function as modulators of late Gl and S phase progression (Pines, 1995). The diminished kinase activity associated with cyclin D1 and CDK4 at 72 h after PH stands in contrast to the finding that the abundance of the cyclin D1/CDK4 complex was highest at this time point (Figure 2a). This suggests that the activity of the cyclin D1/CDK4 complex may be downregulated by CKIs at 72 h after PH. Consistent with previous studies showing that

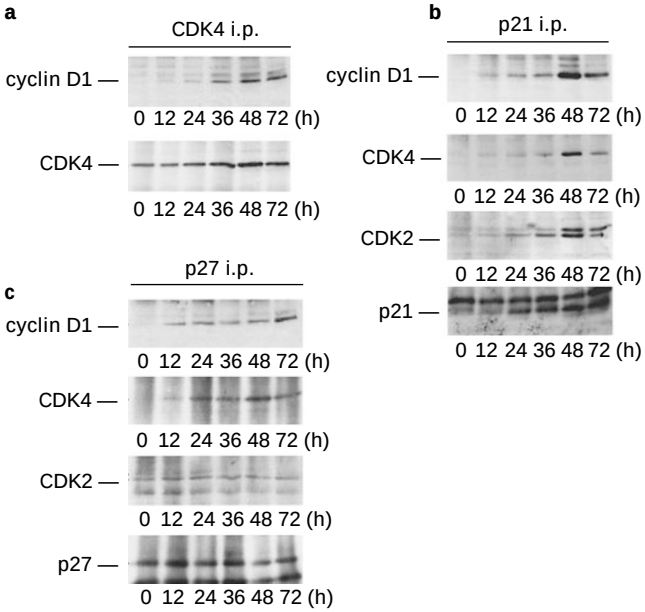

Figure 2 Formation of cyclin/CDK/CKI complexes during liver regeneration. Extracts from liver tissue harvested at the indicated time points after PH in male BALB/c mice were subjected to immunoprecipitation and Western analysis, as described in Materials and methods. (**a**) Cyclin D1/CDK4 complex formation. (**b**) Association of p21 with cyclin D1, CDK4 and CDK2. (**c**) Association of p27 with cyclin D1, CDK4 and CDK2

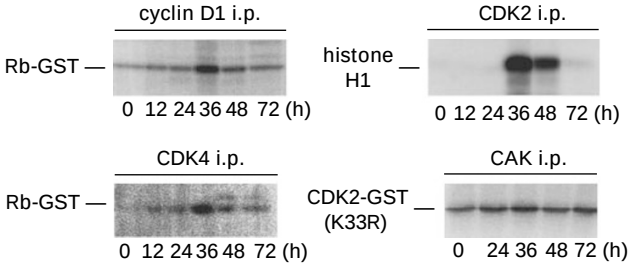

Figure 3 CDK activity after partial hepatectomy. 500 μg of extract from liver tissue harvested at time points from 0 to 72 h after PH in BALB/c mice were subjected to immunoprecipitation with the indicated antibodies. Immune-complex kinase assays were performed as described in Materials and methods

CAK activity is invariant during the cell cycle (Sclafani, 1996; Poon *et al*., 1994), CAK activity was present in quiescent liver and did not vary after PH.

Regulation of CDK2 activity by p27

Even though hepatic p27 levels did not vary substantially during proliferation, it is possible that this protein plays a dynamic role in CDK regulation by redistributing between different cyclin/CDK complexes (Sherr and Roberts, 1995; Poon *et al*., 1995; Soos *et al*., 1996; Reynisdottir *et al*., 1995). To examine the potential role of p27 in the regulation of CDK2 activity in the regenerating liver, a series of mixing experiments was performed (Figure 4). Extracts from BALB/c mouse liver 36 h after PH, containing high CDK2 activity, were mixed with extracts from liver 0, 36 or 72 h after PH. The 0- and 72-h extracts contained little or no detectable CDK2 activity. As shown in Figure 4a, mixing the 36-h extract with the 0-h extract led to 90% inhibition of immunoprecipitable CDK2 activity, while mixing with the 72-h extract resulted in 70% inhibition. As expected, adding 36-h extract to itself led to enhanced CDK2 activity. These results suggest that the 0- and 72-h post-PH liver extracts contained inhibitors of CDK2 activity. In cell culture systems, p27 is heat stable, and boiling cellular extracts leads to the release of 'free' p27 capable of inhibiting CDK activity (Poon *et al*., 1995; Slingerland *et al*., 1994). To test whether a similar heat-stable inhibitory activity was present in regenerating liver, the 0-, 36- and 72-h extracts were boiled for 5 min, and then mixed with the active 36-h extract. In each case, this led to complete inhibition of CDK2 activity, suggesting that a heat-stable CDK inhibitory protein (or proteins) was released by this treatment.

We hypothesized that there was relatively more 'free' p27 in the quiescent (0-h) and postreplicative (72-h) extracts than in the S phase (36-h) extract, and that this excess free p27 could bind and inhibit CDK2-containing complexes. To test this hypothesis, we immunoprecipitated CDK2 after mixing these extracts, and subjected the precipitated proteins to Western blot for p27 (Figure 4b). The amount of p27 immunoprecipitating with CDK2 in the mixed extracts inversely correlated with the histone H1-kinase activity (compare lanes 1–3, Figure 3a and b). This suggests that excess p27 was available in the 0- and 72-h extracts, which inhibited cyclin/CDK2 kinase activity after mixing.

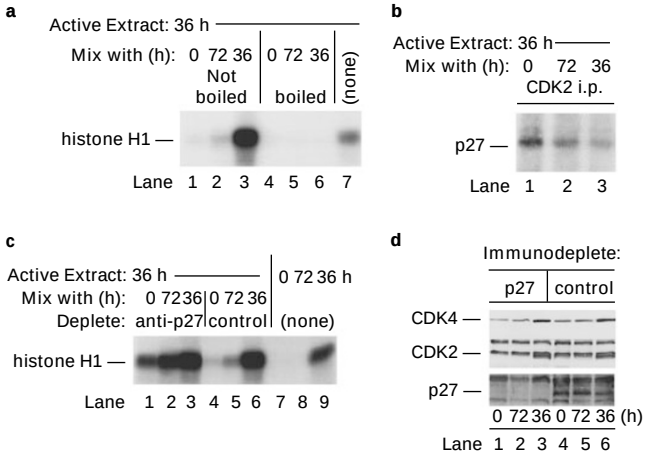

Figure 4 Inhibition of CDK2 activity by p27. (**a**) CDK2 inhibitory activity. 250 μg of an extract from a liver 36 h after PH was mixed with 500 μg of extracts from 0, 72, or 36 h after PH (lanes 1–3), 500 μg of the same three extracts which had been previously boiled for 5 min (lanes 4–6), or an equal volume of buffer alone (lane 7). The samples were mixed gently at 30°C for 30 min, and then CDK2 immune-complex assays were performed as in Figure 3. (**b**) Binding of p27 to CDK2 after mixing. Samples mixed as shown in lanes 1–3 of Figure 4a were subjected to immunoprecipitation with antibody to CDK2, followed by Western blot analysis of the precipitated proteins using an antibody to p27. (**c**) Involvement of p27 in CDK2 inhibition. 250 μg of extract from a liver 36 h after PH was mixed with 500 μg of extracts from 0, 72, or 36 h after PH that had been previously immunodepleted with anti-p27 antibody (lanes 1–3) or a control antibody (lanes 4–6). Mixing and CDK2 kinase assays were carried out as above. The CDK2 activity associated with the 0-, 72- and 36-h extracts (250 μg mixed with buffer alone) is shown in lanes 7–9, respectively. (**d**) p27 immunodepletion. Prior to mixing as shown in Figure 4c, aliquots (50 μg) of the 0-, 72- and 36-h extracts which had been immunodepleted with anti-p27 antibody (lanes 1–3) or a control antibody (lanes 4–6) were subjected to Western blot analysis for p27, CDK2 and CDK4

To further determine whether p27 accounted for some of the CDK2 inhibitory activity in the 0- and 72-h extracts, we immunodepleted p27 from these extracts before mixing (Figure 4c). When the 0-h extract was immunodepleted of p27 before mixing with the active 36-h extract, the amount of CDK2 inhibition was markedly lower. Similarly, when the 72-h extract was immunodepleted of p27 and then mixed with the 36-h extract, 30% more activity was observed than was seen in the 36-h extract alone (compare lanes 2 and 9 of Figure 4c). Immunodepleting p27 from the 36-h extract before mixing had no effect on the resulting CDK2 activity. Western analysis of the immunodepleted extracts (Figure 4d) confirmed that >90% of the p27 was removed by immunodepletion, while CDK4 and CDK2 levels were minimally affected. These results suggest that p27 accounts for some of the CDK2 inhibitory activity present in quiescent (0 h) liver and during the postreplicative (72 h) phase of liver regeneration.

Liver regeneration in p21−/− and p21+/+ mice

To examine whether p21 played a role in hepatocyte proliferation and CDK activity after PH, we conducted studies using a previously established line of p21 knockout mice and cogenic wild-type mice. These mice demonstrate no developmental or pheno-

typic abnormalities, and have no discernible organ abnormalities (Deng *et al*., 1995). However, the effect of p21 deficiency on cell cycle progression and CDK activity in the intact animal has not been directly studied.

We performed PH in female p21+/+ and p21−/− mice and harvested the remnant livers 36, 48 and 72 h later. There were no apparent differences in liver weight or anatomy before PH in the two strains of mice (data not shown). The interval between PH and peak DNA synthesis in rodents is affected by several variables, including species, strain, sex, age and diet (Michalopoulos and DeFrances, 1997; Bucher, 1963). For example, studies in female mice of other strains indicate that peak DNA synthesis occurs 48 h after PH, rather than 36 h, as seen in male BALB/c mice (Paulsen, l990; Lorup, 1977). Consistent with these findings, female p21+/+ mice showed little hepatocyte DNA synthesis as assessed by BrdU uptake at 36 h, but 44.3% BrdU-positive cells were noted at 48 h (Figure 5a). Therefore, in the p21+/+ mice almost all hepatocytes were still in Gl phase at 36 h after PH, while at 48 h a significant proportion had entered S phase. By 72 h after PH, the proportion of BrdU-positive hepatocytes in the p21+/+ mice had declined to 10.3%, consistent with observations in other mouse PH models (Albrecht *et al*., 1994; Cressman *et al*., 1996).

After PH in p21−/− mice, a substantial proportion of hepatocytes were found to be undergoing DNA synthesis at 36 h after PH ($60\pm8\%$ *vs* $2.7\pm3\%$ for the p21+/+ mice; $P<0.05$), indicating that entry into S phase occurred earlier in the p21−/− mice (Figure 5a). At 48 and 72 h after PH, the differences between the p21−/− and p21+/+ mice were not statistically significant. These data suggest that progression through G1 phase of the hepatocyte cell cycle after PH was accelerated in the absence of p21 expression. We then examined biochemical events associated with the transition from G1 phase into S phase in the two groups of mice.

Cell cycle protein expression

No differences in the hepatic expression of CDK4 or p27 after PH was noted in the p21+/+ and p21−/− mice (Figure 5b), and expression of these proteins paralleled the patterns observed in BALB/c mice. The expression of CDK2 was also similar, with the exception that the faster-migrating form of CDK2 appeared at 36 h in the p21−/− mice and at 48 h in the p21+/+ mice. Thus in each group of mice studied, the appearance of the phosphorylated form of CDK2 corresponded to the onset of S phase. Cyclin D1 expression at 36 and 48 h was similar in the two groups of mice, but at 72 h was diminished in the p21−/− mice relative to the p21+/+ mice. We also examined the induction of cyclin A and PCNA, which are upregulated in hepatocytes during S phase (Castro *et al*., 1994; Greenbaum *et al*., 1995). At 36 h after PH, substantially more hepatic cyclin A and PCNA were expressed in the p21−/− than in the p21+/+ mice, further supporting the observation that entry into S phase occurred earlier in the absence of p21.

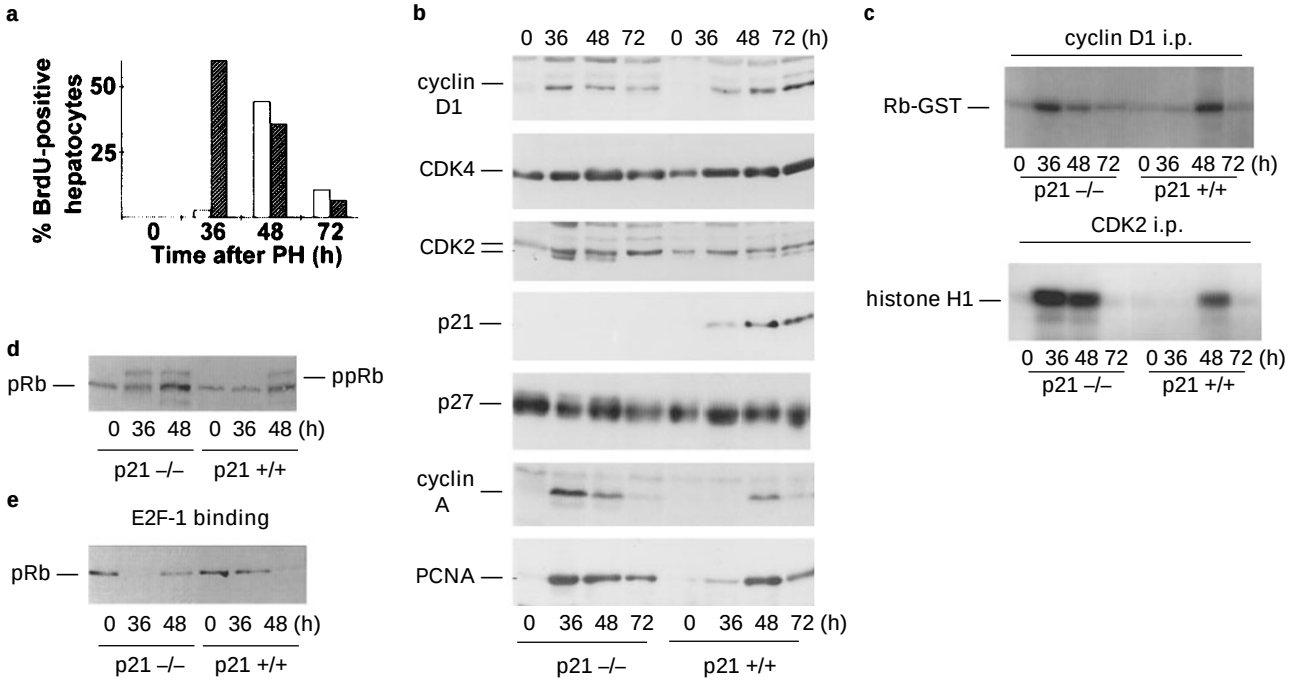

Figure 5 Liver regeneration and cell cycle protein activation in p21−/− and p21+/+ mice. 70% partial hépatectomy (PH) was performed on female p21−/− and p21+/+ mice. (**a**) DNA synthesis after PH. Hepatocyte BrdU uptake was determined at each time point after PH in p21+/+ mice (open bars) and p21−/− mice (crosshatched bars), as described in Materials and methods. (**b**) Cell cycle protein expression after PH. Liver extracts (50 μg) were subjected to Western blot as described in Figure 1, using the indicated antibodies. (**c**) Kinase activation. Cyclin D1- and CDK2-associated kinase activity assays were performed on 500 μg aliquots of liver extracts as shown in Figure 3. (**d**) Retinoblastoma protein expression. Western blot analysis for Rb protein expression was performed on 200 μg aliquots of liver homogenate, as described in Materials and methods. (**e**) E2F-1 binding by Rb. Recombinant E2F-1 bound to agarose beads was incubated with the indicated liver homogenates, followed by recovery of the beads for Western blot analysis using an antibody to Rb, as described in Materials and methods

Cyclin/CDK kinase activity

Since the best-characterized role of p21 is downregulation of cyclin/CDK complex activity, we surmised that activation of G1/S-associated kinase activity would be enhanced in the p21−/− mice. After PH in p21+/+ mice, cyclin D1- and CDK2-associated kinase activity was not substantially induced at 36 h after PH (Figure 5c), consistent with the finding that hepatocytes in these livers were still in G1 phase. At 48 h, corresponding to the rise in hepatocyte BrdU uptake, hepatic cyclin D1- and CDK2-associated kinase activities were markedly elevated. At 72 h, the activity of these enzymes returned to near-basal levels. Thus the results were analogous to the kinase activities observed after PH in the male BALB/c mice: During G1 phase (0–36 h in the p21+/+ mice), kinase activity was not induced, during S phase (48 h) the activity was increased, and during the postreplicative phase (72 h) the activity returned to pre-hepatectomy levels.

In contrast to the p21+/+ mice, the p21−/− mice showed markedly induced hepatic cyclin D1- and CDK2-associated kinase activity at 36 h after PH (Figure 5c). At 48 h after PH, cyclin D1-associated kinase had declined to approximately 50% of peak values, and at 72 h the activity returned to the level of quiescent liver. CDK2 activity remained at near-maximal levels at 48 h after PH in the p21−/− mice, and sharply declined by 72 h. The results indicate that premature activation of cyclin D1- and CDK2-associated kinases during G1 phase of hepatocyte proliferation occurred as a result of p21 deficiency, but the downregulation of these kinases during the postreplicative period did not require p21.

Changes in Rb phosphorylation

Phosphorylation of Rb protein is thought to be a major function of the G1 and S phase CDKs during the cell cycle (Pines, 1995). In quiescent or early G1 phase cells, hypophosphorylated Rb binds and inactivates E2F and related transcription factors. Phosphorylation of Rb by CDKs during late G1 phase causes the release of E2F, leading to transcription of S phase genes and other effects that promote progression through the cell cycle (Grana and Reddy, 1995). To determine whether phosphorylation of Rb occurred during progression into S phase in the regenerating liver, extracts of total protein from p21+/+ and p21−/− mouse liver after PH were subjected to Western blot analysis with an antibody to Rb. As shown in Figure 5d, quiescent liver tissue demonstrated a single form of Rb. After PH in the p21+/+ mice, there was no change in the phosphorylation pattern of Rb at 36 h, but at 48 h a hyperphosphorylated form appeared. In contrast, the hyperphosphorylated form of Rb appeared at 36 h in the p21−/− mice and persisted at 48 h. To further examine whether phosphorylation of Rb corresponded to functional inactivation and loss of E2F binding capacity, liver extracts were incubated with recombinant E2F-1 protein, which was then isolated and subjected to Western blot analysis. As shown in Figure 5e, the faster-migrating form of Rb bound to the recombinant E2F-1 protein in quiescent liver from both strains of mice. At 36 h after PH, the amount of Rb available to bind E2F-1 declined in the p21−/− mice, while a similar decline was noted at 48 h in the p21+/+ mice. These results indicate that the early entry of hepatocytes into S phase at 36 h after PH in the p21−/− mice was accompanied by the premature appearance of a hyperphosphorylated form of Rb and diminished E2F binding capacity of Rb.

Discussion

After 70% PH, most of the remaining hepatocytes replicate once or twice, and then withdraw from the cell cycle (Steer, 1995; Michalopoulos and DeFrances, 1997). In this model, which represents one of the most striking examples of controlled cell proliferation *in vivo*, a precise balance between proliferative and antiproliferative signals results in accurate restoration of liver mass. Although significant insight has been gained into the factors that promote hepatocyte proliferation, relatively little is known about mechanisms that inhibit hepatocyte cell cycle progression in the regenerating liver (Fausto *et al*., 1995; Michalopoulos and DeFrances, 1997). Because the CKIs are implicated in multiple growth-inhibitory pathways in cell culture systems, we examined the role of p21 and p27 in the process of liver regeneration. The results suggest that the expression of p21 and p27 in the regenerating liver is different than in cell culture systems, and that these proteins regulate hepatocyte CDK activity during post-natal liver growth. These studies also indicate that hepatocytes progress through G1 phase more rapidly in the absence of p21, suggesting that modulation of G1-associated cyclin/CDK activity by p21 is an important determinant of the prereplicative stage of liver regeneration.

According to current models of liver regeneration, progression of hepatocytes through G1 phase is mediated by cyclins and CDKs, but little is known about the regulation of hepatocyte cyclin/CDK activity (Fausto *et al*., 1995; Cressman *et al*., 1996). Several studies suggest that the expression of cyclins, CDKs, and related genes in the regenerating liver differs from that observed in cell culture systems. For example, the expression of cyclin and CDK mRNAs during liver regeneration is regulated primarily at the post-transcriptional level by changes in mRNA stability, while transcriptional regulation plays an important role in other systems (Kren and Steer, 1996). Furthermore, expression of cyclin D1 protein in the regenerating rat liver is uncoupled from that of its mRNA, a phenomenon that is seen in some but not all cell types in culture (Loyer *et al*., 1994; Albrecht *et al*, 1995). Thus, findings from the abundant literature on cell cycle control in tissue culture systems cannot necessarily be extrapolated to the regenerating liver *in vivo*.

Expression of cyclin D1 is thought to be a marker of progression through late G1 phase in hepatocytes (Albrecht *et al*., 1995; Cressman *et al*., 1996; Loyer *et al*., 1996; Yamada *et al*., 1997), but previous studies have not documented activation of cyclin D1- or CDK4-associated kinase activity during the process of liver regeneration. In other cell lines, activation of cyclin D1/CDK4 is a critical step during the growth-factor-mediated progression through the G1 restriction point (Pines, 1995). The cyclin D1/CDK4 complex is thought

to be the focal point of signal transduction pathways that integrate growth-stimulatory and -inhibitory signals into the cell cycle machinery (Grana and Reddy, 1995; Sherr, 1996). Thus the present finding that cyclin D1/CDK4 kinase activity was upregulated during S phase (but not early G1 phase) of liver regeneration is consistent with the concept that activation of this complex plays an important role in progression through the G1-S transition.

The kinase activity associated with cyclin D1, CDK4, and CDK2 was diminished in the postreplicative phase of liver regeneration (72 h after PH), corresponding to diminished hepatocyte proliferation at this time point. In the case of CDK2, diminished kinase activity at 72 h may have resulted in part from decreased levels of cyclin A. For cyclin D1/CDK4, however, the abundance of this complex was increased at 72 h while the activity of the kinase was diminished. Downregulation of cyclin/CDK activity could be due to several mechanisms, including changes in the phosphorylation status of the CDKs (Morgan, 1995). For full activation of cyclin/CDK complexes to occur, the CDK must be phosphorylated on a threonine residue near the active site, which is carried out by CAK (Sclafani, 1996). In the current study, the appearance of a faster-migrating form of CDK2 corresponded to peak activity of this kinase, and suggests that phosphorylation of CDK2 at Thrl60 by CAK was enhanced during S phase in this model. However, CAK activity was invariant after PH, consistent with the observation that the activity of this enzyme complex (cyclin H/CDK7) does not change during the cell cycle (Poon *et al*., 1994). This discrepancy could possibly be due to binding of other proteins to CDK2 that affect its phosphorylation by CAK; for example, the rate of this phosphorylation can be affected by cyclin, p21, or p27 binding to the target CDK2 (Morgan, 1995; Sclafani, 1996). However, we did not directly study the regulation of endogenous CDKs by CAK, the Wee 1 kinase, or the Cdc25 family of phosphatases. Nor did we study each of the potential cyclin/CDK combinations in the regenerating liver. Rather, we focused our efforts on the best-characterized inhibitors of cyclin/CDK activity, p21 and p27.

To examine the potential role of p21 and p27 in the regulation of CDK activity and hepatocyte proliferation in the regenerating liver, we first examined the expression of these proteins. p21 protein was undetectable by Western analysis in quiescent mouse liver. The level of p21 protein increased steadily after PH and peaked in the postreplicative period. This is in contrast to the expression of its mRNA, which is induced in a biphasic manner after PH (Albrecht *et al*., 1997). Similar uncoupling of protein and mRNA expression has been observed for other genes in the regenerating liver (Albrecht *et al*., 1995; Greenbaum *et al*., 1995), and suggests regulation at the level of translation or protein stability. Regulation of p21 expression at the level of protein stability has been recently documented (Blagosklonny *et al*., 1996); the stability of p21 protein can be enhanced by the transcription factor c/EBPα, leading to growth inhibition in fibrosarcoma cells (Timchenko *et al*., 1996). Therefore, altered protein stability during liver regeneration might explain the discordance between p21 mRNA and protein, but this mechanism requires further examination. Previous studies have indicated p53-dependent and -independent regulation of p21 mRNA expression in the liver (Albrecht *et al*., 1997; Serfas *et al*., 1997), and the current findings further suggest that the regulation of hepatic p21 occurs at multiple levels.

In other cell types, the level of p27 decreases as cells leave G0 and enter the cell cycle (Poon *et al*., 1995; Agrawal *et al*., 1996). The presence of p27 in G0 and early G1 phase serves to inactivate cyclin/CDK complexes at these stages (Poon *et al*., 1995; Soos *et al*., 1996); antisense inhibition of p27 allows mitogen-independent growth of BALB/c-3T3 cells, indicating that the abundance of p27 relative to the G1 cyclin/CDK complexes is an important determinant of the G1 restriction point in these cells (Coats *et al*., 1996). In contrast to cells in culture, the level of p27 did not decline as hepatocytes entered the cell cycle in the regenerating liver. However, this does not rule out the possibility that p27 plays a dynamic role in CDK regulation, because it can redistribute between different cyclin/CDK complexes during cell cycle progression. For example, during G1 phase progression in Swiss 3T3 fibroblasts, accumulation of cyclin D1/CDK4 results in more p27 binding to this complex, leaving less p27 to bind to CDK2 (Poon *et al*., l995). In G0, the absence of cyclin D1/CDK4 complexes leaves more p27 available to bind CDK2. Similarly, binding of INK4-type inhibitors can displace p27 from cyclin D/CDK4 complexes, leading to inhibition of CDK2 (Sherr and Roberts, 1995; Reynisdottir *et al*., 1995).

The present study suggests that p27 may act as an inhibitor of CDK2 activity in the pre- and postreplicative phase of liver regeneration. The mixing experiments demonstrated that extracts from quiescent or postreplicative liver contain an inhibitor (or inhibitors) of active cyclin/CDK2 complexes from the S phase extract (Figure 4a). The inhibitor was heat-stable and was increased in efficiency by boiling the extracts, both of which are characteristic of p27 (Poon *et al*., 1995; Slingerland *et al*., 1994). More p27 was available to bind CDK2 in the quiescent and postreplicative extracts than in the S phase extract (Figure 4b). Immunodepletion of p27 before mixing essentially eliminated the inhibitory factor(s) from quiescent liver extract, suggesting that p27 accounted for a significant proportion of the CDK2 inhibitory activity in quiescent liver (Figure 4c). Accumulation of cyclin D1/CDK4 complexes during the prereplicative phase after PH was associated with increased binding of p27 to these proteins (Figure 2c). These data are consistent with the model that p27 is 'free' in G0 and early G1 phase, but as cyclin D/CDK4 complexes increase in abundance during late G1 phase, they sequester p27 and prevent inhibition of cyclin/CDK2 complexes (Sherr and Roberts, 1995; Poon *et al*., 1995; Polyak *et al*., 1994). Immunodepletion of p27 from postreplicative liver extracts before mixing also eliminated the inhibition of CDK2 and in fact led to enhanced activity, possibly due to rearrangement of cyclin/CDK/CKI proteins in the mixed extracts. This suggests that p27 plays a role in downregulating CDK2 activity in the postreplicative phase of liver regeneration. If cyclin D1/CDK4 complexes do sequester p27 in the early phases of liver regeneration, then it is possible

that INK4-type inhibitors displace p27 from these complexes in the postreplicative phase (Sherr and Roberts, 1995; Reynisdottir *et al*., 1995). The potential involvement of p27 and INK4 inhibitors in the regenerating liver requires further study.

We chose to take a different approach to study the role of p21 in the regenerating liver, by comparing the response to PH in cogenic p21−/− and p21+/+ mice. The results indicate that hepatocytes progressed more rapidly through G1 phase in the absence of p21. In the p21−/− mice, most hepatocytes were in S phase at 36 h after PH, compared to less than 3% of hepatocytes in the p21+/+ mice. Further biochemical evidence of accelerated G1 progression in the p21−/− mice included premature activation of cyclin D1- and CDK2-associated kinases, induction of PCNA and cyclin A, and Rb hyperphosphorylation. Although we did not exhaustively study time points in the pre- and postreplicative phases of liver regeneration in these mice, these data are sufficient to indicate a shortened G1 interval in the p21−/− mice.

In cell culture systems, the CKIs are thought to set a threshold of CDK inhibition in G0 and early G1 phase. According to this model, the abundance of G1 cyclin/CDK complexes must exceed this threshold for progression through the G1 restriction point and into S phase (Sherr and Roberts, 1995). Other *in vitro* data suggest that p21 promotes assembly of active cyclin/CDK complexes, and thus may facilitate progression through Gl phase (Zhang *et al*., 1994; LaBaer *et al*., 1997). These two models could be reconciled by the possibility that the relative ratio of p21 (or other Cip/Kip family members) in each cyclin/CDK complex determines its kinase activity; the presence of one CKI per complex allows activation, while two CKIs is inhibitory. During hepatocyte proliferation in the regenerating liver, a high ratio of CKIs relative to cyclin/CDK complexes would be expected to suppress kinase activity. We hypothesize that during late G1 phase the abundance of cyclin/CDK complexes must exceed the threshold set by the CKIs in order for hepatocyte proliferation to occur. Our data support the model that induction of p21 during G1 phase serves to raise the threshold of CDK inhibition and delay the onset of S phase; in the p21−/−mice this threshold was lower, leading to earlier entry into S phase.

The effect of gene deletions in knockout mice can be diminished by compensatory changes in the expression of other proteins (Weintraub, 1993). However, since p21 is not expressed in normal adult mouse or human liver (el-Deiry *et al*., 1995; Albrecht *et al*., 1997), no compensatory changes in gene expression would be expected in quiescent liver. The fact that we observed accelerated G1 progression in the p21−/− mice suggests that functionally redundant genes were not able to fully compensate for p21 deficiency during the prereplicative phase of regeneration. In the postreplicative phase, downregulation of cyclin D1- and CDK2-associated kinases occurred in both p21−/− and p21+/+ mice, suggesting that p21 is not involved in this downregulation, or that the expression of other genes compensated for p21 deficiency. For example, diminished cyclin D1 expression at 72 h after PH in the p21−/− mice (relative to the p21+/+ mice) may reflect an adaptive change in gene expression to allow downregulation of CDK activity in the postreplicative period. However, this study was not designed to fully characterize the regulation of CDKs and hepatocyte proliferation in the postreplicative phase of liver regeneration. Other authors have proposed that the liver possesses redundant systems to inhibit further liver regeneration once liver mass has been restored (Michalopoulos and DeFrances, 1997). Thus, while it is tempting to speculate that the enhanced p21 expression seen in the postreplicative phase (72 h after PH) in normal mice inhibits CDK activity and further hepatocyte replication, more study is needed to define the role of p21 and other CKIs at this stage of liver regeneration.

The data presented here suggest that both p2l and p27 are involved in hepatocyte growth regulation during liver regeneration. The evidence is strongest for p21, since our data indicate that its absence leads to more rapid progression through G1 phase of the cell cycle, suggesting that this protein plays a growth-inhibitory role. This complements the work of Wu *et al*. demonstrating that transgenic mice with forced hepatic expression of p21 have markedly impaired regeneration after PH (Wu *et al*., 1996). Taken together, these studies suggest that the intracellular level of p21 regulates hepatocyte G1 phase progression, and indeed may determine whether cells proceed through the G1 restriction point and ultimately proliferate. Future studies using mice deficient in CKIs and other growth-inhibitory proteins are likely to provide insight into the molecular control of hepatocyte proliferation *in vivo*.

Materials and methods

Animals

Male BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Female p21−/− and p21+/+ 129/SvEvTacfBR mice have been previously described (Deng *et al*., 1995). At 8 weeks of age, standard 70% PH was performed by a single investigator (JHA), as previously described (Albrecht *et al*., 1997); mortality was less than 5% for this procedure. Animals were provided food and water *ad libitum*. At the indicated time points, animals were sacrificed and the remnant liver was harvested, frozen in liquid nitrogen, and stored at −80°C until further use. Protein-deprived animals were provided only 10% glucose solution for 48 h prior to sacrifice. The p21−/− and p21+/+ mice were given 50 mg/kg of bromodeoxyuridine (BrdU; Amersham, Arlington Heights, IL) by intraperitoneal injection 2 h prior to sacrifice.

Antibodies and reagents

The following antibodies were used for these experiments: Rabbit polyclonal anti-CDK2 (SC-163; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-CDK4 (SC-260; Santa Cruz Biotechnology), mouse monoclonal anti-cyclin D1 (SC-450; Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin D1 (06-137; Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-cyclin E (SC-481; Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin A (a generous gift from Dr Edward Leof), mouse monoclonal anti-cyclin A (E23; a generous gift from Dr Julian Gannon), rabbit polyclonal anti-p21 (a generous gift from Dr Claudio Schneider), mouse mono-

clonal anti-p21 (Ab-4; Oncogene Science, Cambridge, MA), rabbit polyclonal anti-p27 (SC-528; Santa Cruz Biotechnology), mouse monoclonal anti-p27 (K25020; Transduction Laboratories, Lexington, KY), mouse monoclonal anti-PCNA (SC-56; Santa Cruz Biotechnology), mouse monoclonal anti-retinoblastoma protein (14001A; Pharmigen, San Diego, CA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA), and HRP-conjugated goat anti-mouse IgG (Sigma Chemical Co, St Louis, MO). All other reagents were molecular biology grade and obtained from Sigma or Boerhinger Mannheim (Indianapolis, IN).

Preparation of cellular extracts and Western analysis

Whole mouse liver was homogenized in a modified Tween-20 buffer (50 mM HEPES, pH 7.5, l50 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM NaF, 7.5 mM EGTA, 7.5 mM $MgCl_2$) or Triton-X-100 buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton-X-100) containing protease and phosphatase inhibitors (1 mM Na_3VO_4, 25 mM β-glycerolphosphate, 87 μg/mL phenylmethylsulfonyl fluoride, 4 μg/mL N-α-Tosyl-L-lysine chloromethyl ketone, 1 μg/mL aprotinin, 0.1 μg/mL leupeptin, 1 μg/mL pepstatin, and 5 mM DTT). The homogenates were sonicated and clarified by centrifugation for 10 min at 15 000 r.p.m. The supernatants were aliquoted and stored at -80°C. Protein concentration was measured using the BioRad DC protein reagent (BioRad, Hercules, CA) following the manufacturer's protocol. For Western analysis, 50 μg of protein extract per lane was added to an equal volume of 2 × Llammeli sample buffer and was size-fractionated on 12 or 15% SDS–PAGE, transferred to nitrocellulose, and detected using the ECL Chemiluminescence Kit (Amersham, Arlington Heights, IL)

Immunoprecipitation/Western blot analysis

Liver extracts prepared in Triton-X-100 buffer (500 μg) were immunoprecipited with appropriate antibody (1 μg per sample), and incubated with gentle rocking for 1–4 h. Precipitated proteins were collected with protein A/G beads (Oncogene Science, Camblidge, MA), washed, resuspended in 2 × sample buffer and resolved by 12% SDS–PAGE. Transfer and blotting was as described above.

Northern Analysis

Northern analysis of p21 and albumin expression was performed on total RNA isolated from BALB/c mouse liver, as previously described (Albrecht *et al*., 1997).

Immunoprecipitation/kinase assay

Liver was homogenized in Tween-20 buffer as above. 500 μg of liver extract was incubated with 1 μg of anti-CDK2 or anti-CDK4 antibody at 4°C for 2–6 h with gentle rocking. Precipitates were recovered on protein A/G beads as above. Immune complexes were washed three times in bead buffer (50 mM Tris-Cl pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40 with protease and phosphatase inhibitors) and twice in kinase buffer A (80 mM β-Glycerol-phosphate, 20 mM EGTA, 15 mM $Mg(OAc)_2$, 1 mM DTT, 50 μM ATP). For CDK2 kinase assays, the beads were resuspended in 10 μL kinase buffer A containing 5 μCi [γ^{32}P]ATP (3000 Ci/mmol; Amersham, Arlington, IL) and 1 μg histone H1. For CDK4 kinase assays, 1 μg retinoblastoma protein-GST (Rb-GST) fusion protein was substituted for histone H1. The Rb-GST protein was prepared from a plasmid generously provided by Dr Jean Wang (Lin and Wang, 1992), using glutathione-agarose beads (Pharmacia, Piscataway, NJ), as described by the manufacturer. For cyclin D1 kinase assays, 500 μg liver extracts were incubated with 2 μg of anti-cyclin D1 antibody (SC-450), and immune complexes collected as above. Protein A/G beads were washed four times in 50 mM HEPES pH 7.5, 1 mM DTT and four times in kinase buffer B (50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, and 1 mM NaF). Beads were resuspended in 30 μl of kinase buffer B containing 1 μg of Rb-GST, 20 μM ATP, and 10 μCi [γ^{32}P]ATP. Kinase assays were incubated at room temperature for 40 min, and reactions were terminated by the addition of 2 × sample buffer. CAK assays were performed on 500 μg of liver homogenates, as previously described (Poon *et al*., 1994). Samples were subsequently subjected to 12% SDS–PAGE, and ^{32}p-labeled proteins were detected and quantitated with the PhosphorImager SI and ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

Mixing experiments

Liver extracts prepared in Tween-20 buffer were mixed as indicated in Figure 4, and then incubated at 30°C for 30 min. Some extracts were boiled for 5 min, clarified by centrifugation, and cooled on ice prior to mixing. Other extracts were immunodepleted with protein A/G beads precoated with anti-p27 antibody (SC-528) or an irrelevant control antibody three successive times before mixing. After mixing, the samples were subjected to immunoprecipitation, Western blot analysis, and CDK2 kinase assays, as described above.

Rb immunoblotting and E2F-1 binding assay

Liver tissue was homogenized in NET-N buffer (100 mM NaCl, 1 mM EDTA, 100 mM Tris-Cl, pH 8.0, 0.5% Nonidet P-40) supplemented with protease and phosphatase inhibitors. For immunoblotting, 200 μg of this homogenate per lane was mixed with 2 × Llammeli sample buffer and subjected to 8% SDS–PAGE and immunoblotting as above. E2F-1 binding assays were performed essentially as described (Knudsen and Wang, 1996; Chen *et al*., 1996). In brief, recombinant E2F-1-GST fusion protein was expressed and purified as above, using a plasmid generously provided by Dr Eric Knudsen. Approximately 250 ng of E2F-1-GST immobilized on glutathione-agarose beads was added to 1.5 mg of liver homogenate in NET-N and incubated with gentle rocking at 4°C for 2 h. The beads were collected by centrifugation, washed in NET-N, and resuspended in 2 × sample buffer. The samples were then subjected to SDS–PAGE and Western analysis for Rb, as described above.

BrdU uptake in regenerating liver

After harvest of tissue from p21−/− and p21+/+ mice, liver biopsies were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (5 μM) were prepared and mounted on glass slides. Immunohistochemical staining for BrdU was performed with the Amersham Cell Proliferation Kit, according to the manufacturer's instructions. Labeling indices were determined by counting BrdU-positive nuclei in a minimum of 300 hepatocytes per sample. The mean percentage of BrdU-positive hepatocytes was determined for at least three animals at each time point after PH in the p21+/+ and p21−/− mice. Statistical analysis was performed using the Mann-Whitney U test.

Acknowledgements
The authors gratefully acknowledge the following investigators for generously providing reagents used in these studies: Julian Gannon, Eric Knudsen, Edward Leof, Claudio Schneider, Bert Vogelstein, and Jean Wang. This work was supported by grants from the American Liver Foundation, Hennepin Faculty Associates, and the Edith H Lynum Trust.

References

Agrawal D, Hauser P, McPherson F, Dong F, Garcia A and Pledger WJ. (1996). *Mol. Cell. Biol.,* **16,** 4327–4336.
Akashi M, Hachiya M, Osawa Y, Spirin K, Suzuki G and Koeffler HP. (1995). *J. Biol. Chem.,* **270,** 19181–19187.
Albrecht JH, Hoffman JS, Kren BT and Steer CJ. (1994). *FEBS Lett.,* **347,** 157–162.
Albrecht JH, Hu MY and Cerra FB. (1995). *Biochem. Biophys. Res. Commun.,* **209,** 648–655.
Albrecht JH, Meyer AH and Hu MY. (1997). *Hepatology,* **25,** 557–563.
Blagosklonny MV, Wu GS, Omura S and el-Deiry WS. (1996). *Biochem. Biophys. Res. Commun.,* **2,** 564–569.
Bucher NLR. (1963). *Int. Rev. Cytol.,* **15,** 245–300.
Castro A, Jaumot M, Verges M, Agell N and Bachs O. (1994). *Biochem. Biophys. Res. Commun.,* **201,** 1072–1077.
Chen Y, Knudsen ES and Wang JYJ. (1996). *J. Biol. Chem.,* **271,** 19637–19640.
Coats S, Flanagan WM, Nourse J and Roberts JM. (1996). *Science,* **272,** 877–880.
Cornell RP, Liljiquist BL and Bartizal KF. (1990). *Hepatology,* **11,** 916–922.
Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V and Taub R. (1996). *Science,* **274,** 1379–1383.
Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y and Wang XF. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 5545–5549.
Deng C, Zhang P, Harper JW, Elledge SJ and Leder P. (1995). *Cell,* **82,** 675–684.
el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parson R, Trent JM, Lin D, Mercer E, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.
el-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR, Kinzler KW and Vogelstein B. (1995). *Cancer Res.,* **55,** 2910–2919.
Fausto N, Laird AD and Webber EM. (1995). *FASEB J.,* **9,** 1527–1536.
Fero M, Rivkin M, Tasch M, Porter P, Carow C, Polyak K, Tsai L-H, Broundy V, Perlmutter R, Kaushansky K and Roberts J. (1996). *Cell,* **85,** 733–744.
Grana X and Reddy EP. (1995). *Oncogene,* **11,** 211–219.
Greenbaum LE, Cressman DE, Haber BA and Taub R. (1995). *J. Clin. Invest.,* **96,** 1351–1365.
Grisham JW. (1962). *Cancer Res.,* **22,** 842–849.
Guo K, Wang J, Andres V, Smith RC and Walsh K. (1995). *Mol. Cell. Biol.,* **15,** 3823–3829.
Haber BA, Mohn KL, Diamond RH and Taub R. (1993). *J. Clin. Invest.,* **91,** 1319–1326.
Kato JY, Matsuoka M, Polyak K, Massague J and Sherr CJ. (1994). *Cell,* **79,** 487–496.
Kiyokawa H, Kineman R, Manova-Todorova K, Soares V, Hoffman E, Ono M, Khanam D, Hayday A, Frohman L and Koff A. (1996). *Cell,* **85,** 721–732.
Knudsen ES and Wang JYJ. (1996). *J. Biol. Chem.,* **14,** 8313–8320.
Kren BT and Steer CJ. (1996). *FASEB J.,* **10,** 559–573.
LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A and Harlow E. (1997). *Genes & Dev.,* **11,** 847–862.
Li CY, Suardet L and Little JB. (1995). *J. Biol. Chem.,* **270,** 4971–4974.
Lin BYT and Wang JYJ. (1992). Regulation of the Eukaryotic cell cycle. Ciba Foundation Symposium. Wiley: New York, pp. 227–243.
Lorup C. (1977). *Cell. Tissue Kinet.,* **10,** 477–485.
Loyer P, Glaise D, Cariou S, Baffet G, Meijer L and Guguen-Guillouzo C. (1994). *J. Biol. Chem.,* **269,** 2491–2500.
Loyer P, Cariou S, Glaise D, Bilodeau M, Baffet G and Guguen-Guillouzo C. (1996). *J. Biol. Chem.,* **271,** 11484–11492.
Lu XP, Koch KS, Lew DJ, Dulic V, Pines J, Reed SI, Hunter T and Leffert HL. (1992). *J. Biol. Chem.,* **267,** 2841–2844.
Luo Y, Hurwitz J and Massague J. (1995). *Nature,* **375,** 159–161.
Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B and Jacks T. (1995). *Genes & Dev.,* **9,** 935–944.
Marx J. (1995). *Science,* **267,** 963–964.
Mead JE, Braun L, Martin DA and Fausto N. (1990). *Cancer Res.,* **50,** 7023–7030.
Michalopoulos GK and DeFrances MC. (1997). *Science,* **276,** 60–66.
Missero C, Di Cunto F, Kiyokawa H, Koff A and Dotto GP. (1996). *Genes & Dev.,* **10,** 3065–3075.
Morgan DO, (1995). *Nature,* **374,** 131–134.
Nakanishi M, Adami GR, Robertoye RS, Noda A, Venable SF, Dimitrov D, Pereira-Smith OM and Smith JR. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4352–4356.
Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I and Loh D. (1996). *Cell,* **85,** 707–720.
Noda A, Ning Y, Venable SF, Pereira-Smith OM and Smith JR. (1994). *Exp. Cell. Res.,* **211,** 90–98.
Pardee AB. (1989). *Science,* **246,** 603–608.
Paulsen JE. (1990). *In vivo,* **4,** 235–238.
Pines J. (1995). *Biochem., J.* **308,** 697–711.
Polyak K, Kato J, Solomon MJ, Sherr CJ, Massague J, Roberts JM and Koff A. (1994). *Genes & Dev.,* **8,** 9–22.
Poon RYC, Yamashita K, Howell M, Ershler MA, Belyavsky A and Hunt T. (1994). *J. Cell Sci.,* **107,** 2789–2799.
Poon RYC, Toyoshima H and Hunter T. (1995). *Mol. Biol. Cell,* **6,** 1197–1213.
Reynisdottir I, Polyak K, Iavarone A and Massague J. (1995). *Genes & Dev.,* **9,** 1831–1845.
Sclafani RA. (1996). *Curr. Opin. Cell Biol.,* **8,** 788–794.
Serfas MS, Goufamn E, Feuderman MH, Gartel AL and Tyner AL. (1997). *Cell Growth Differ.,* **8,** 951–961.
Sherr CJ and Roberts JM. (1995). *Genes & Dev.,* **9,** 1149–1163.
Sherr CJ. (1996). *Science,* **274,** 1672–1677.
Shim J, Lee H, Park J, Kim H and Choi EJ. (1996). *Nature,* **381,** 804–806.
Slingerland JM, Hengst L, Pan CH, Alexander D, Stampfer MR and Reed SI. (1994). *Mol. Cell. Biol.,* **14,** 3683–3694.
Soos TJ, Kiyokawa H, Yan JS, Rubin MS, Giordano A, DeBlasio A, Bottega S, Wong B, Mendelsohn H and Koff A. (1996). *Cell Growth Differ.,* **7,** 135–146.
Steer CJ. (1995). *FASEB J.,* **9,** 1396–1400.
Timchenko NA, Wilde M, Nakanishi M, Smith JR and Darlington GJ. (1996). *Genes & Dev.,* **10,** 804–815.
Waga S, Hannon GJ, Beach D and Stillman B. (1994). *Nature,* **369,** 574–578.
Weintraub H. (1993). *Cell,* **75,** 1241–1244.
Wu H, Wade M, Krall L, Grisham J, Xiong X and Van Dyke T. (1996). *Genes & Dev.,* **10,** 245–260.
Yamada Y, Kirillova I, Peschon JJ and Fausto N. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 1441–1446.
Zhang H, Hannon GJ and Beach D. (1994). *Genes & Dev.,* **8,** 1750–1758.