Regulation of G₁ cyclin-dependent kinases in the liver: role of nuclear localization and p27 sequestration

Jeffrey H. Albrecht, ¹,² Brenda M. Rieland, ² Christopher J. Nelsen, ² and Cory L. Ahonen ²

¹Department of Medicine, Hennepin County Medical Center, Minneapolis 55415; and ²Minneapolis Medical Research Foundation, Minneapolis, Minnesota 55404

Cell proliferation is regulated by extracellular factors acting primarily during the prereplicative (G₁) phase of the cell cycle. Growth-stimulatory and -inhibitory signals arising from extracellular stimuli converge on cell cycle control proteins that determine whether cells progress through G₁ phase. The major components of the cell cycle machinery are the cyclins and cyclin-dependent kinases (Cdks), which form active kinase complexes during specific portions of the cell cycle. The activity of the cyclin/Cdk complexes are regulated by numerous intracellular proteins, including Cdk inhibitors that oppose cell cycle progression. Because control of cell proliferation is accomplished primarily during G₁ phase, significant attention has been directed toward the cyclin/Cdk complexes active during this stage of the cell cycle. The D-type cyclins (primarily cyclin D₁) are induced by mitogens and along with their major kinase partners, Cdk4 and Cdk6, play a central role in regulating G₁ progression. The cyclin E/Cdk2 complex also plays an essential part in the transition from G₁ into S phase. The best-characterized substrates of the G₁ cyclin/Cdk complexes are the retinoblastoma protein (Rb) and the related p107 and p130 proteins.

Activation of cyclin/Cdk complexes during G₁ requires several steps. In the case of cyclin D₁/Cdk4, Cdk4 is constitutively expressed in most cells, whereas cyclin D₁ is upregulated by mitogens during mid- to late G₁ phase. Full activation of the cyclin D₁/Cdk4 complex requires phosphorylation of Cdk4 at Thr-172, which is catalyzed by the Cdk-activating kinase (CAK). Mammalian CAK appears to be comprised of cyclin H/Cdk7, although other kinases may be capable of performing this function. The activity of cyclin D₁/Cdk4 is enhanced by dephosphorylation of the Cdk at Tyr-17 by the Cdc25 family of phosphatases, whereas phosphorylation of this residue by the Wee1 kinase inhibits activation. Cyclin D₁/Cdk4 activity is negatively regulated by Cdk inhibitors, which consist of two major families. The Ink4 inhibitors (p15, p16, p18, and p19) specifically inhibit Cdk4 and Cdk6 by preventing complex formation with the D-type cyclins. The Cip/Kip family of proteins (p21, p27, and p57) bind and inhibit numerous cyclin/Cdk complexes. Complexes containing D-type cyclins require higher stoichiometric concentrations of Cip/Kip proteins for kinase inhibition than do Cdk2-containing complexes. Furthermore, trimeric complexes containing cyclin D, Cdk4, and Cip/Kip inhibitors have been shown to possess Rb kinase activity.

Liver regeneration, or compensatory hyperplasia of the liver, is a well-described adaptive response to hepatic injuries that results in the loss of functional hepatic mass. In the best-studied model of liver regeneration, that of 70% partial hepatectomy (PH) in...
rodents, liver mass and function is precisely restored within 7–10 days. In the first 1–2 days after PH, a large population of hepatocytes progresses through the cell cycle in a relatively synchronous manner. The PH model therefore represents a unique in vivo model of highly regulated cell cycle progression.

Prior studies have documented the expression and activation of cyclin/Cdk complexes in regenerating rodent liver after PH (4, 7, 16, 22, 29, 30, 44, 57, 59). Consistent with abundant data from tissue culture systems demonstrating that cyclin D1 governs progression through G1 phase of the cell cycle, cyclin D1 has been proposed to be a key regulator of hepatocyte proliferation. Cyclin D1 is induced during the prereplicative phase in the regenerating liver after PH and is upregulated in human liver specimens demonstrating evidence of regeneration (3). In several models, impaired liver regeneration after PH is associated with diminished cyclin D1 expression (2, 10, 62). In primary hepatocytes in culture, cyclin D1 is induced by growth factor at time points corresponding to the G1 restriction point (3, 28), and transfection of hepatocytes with cyclin D1 leads to mitogen-independent cell cycle progression (1).

There are few data regarding the regulation of cyclin D1 and associated kinase activity during hepatocyte proliferation in vivo. Upregulation of cyclin D1 protein after PH is associated with formation of cyclin D1/Cdk4 complexes and associated Rb kinase activity (4, 22). However, previous studies have not clearly delineated the regulation of cyclin D1/Cdk4 and other G1-associated kinase complexes by Cdk-inhibitory proteins, changes in phosphorylation, or intracellular trafficking. The purpose of the current study was to examine potential mechanisms by which these complexes are regulated in the regenerating liver.

MATERIALS AND METHODS

Materials. Young adult male Sprague-Dawley rats (200–225 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Polyclonal antibodies used in this study included the following: cyclin A (sc-596, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin A (a gift of Dr. Edward Leof), cyclin D1 (06-137, Upstate Biotechnology, Lake Placid, NY), cyclin D3 (sc-182, Santa Cruz), cyclin E (sc-481, Santa Cruz), Cdk2 (sc-163, Santa Cruz), Cdk4 (sc-260, Santa Cruz), p21 (sc-397G, Santa Cruz), p27 (a gift of Dr. Deepak Agrawal), p57 (sc-1037G, Santa Cruz), p107 (sc-318 Santa Cruz), p130 (sc-317, Santa Cruz), and rat laminin (a gift of Dr. Scott Kaufmann). The monoclonal antibodies included the following: Cdk7 (Zymed, San Francisco, CA), cyclin D1 (72-136G, Zymed), cyclin D3 (DCS22, Neomarkers), cyclin D3 (sc-453, Santa Cruz), and p27 (Transduction Laboratories, Lexington, KY). Baculoviruses encoding cyclin E-glutathione S-transferase (GST) and Cdk2 were a gift of Dr. Boris Sarcevic (43).

Purified recombinant cyclin H/Cdk7/MAT1 complexes produced by baculoviruses were a gift of Dr. Robert Fisher (17). The plasmid encoding the cyclin D1-GST fusion protein was a gift of Dr. Charles Sherr. The plasmid encoding a Rb-GST fusion protein was a gift of Dr. Jean Wang. The plasmids encoding Cdc25B-GST, Cdk2-GST, and cyclin A-histadine were gifts of Dr. Randy Poon (39, 41). The plasmid encoding Cdc25A-GST was a gift of Dr. Helen Piwnica-Worms. Protein A and glutathione Sepharose 4B beads were obtained from Pharmacia (Piscataway, NJ). SF9 cells and TNM-FH insect medium were obtained from Pharmingen (San Diego, CA). Protein A/G beads were from Calbiochem (San Diego, CA). The DC protein reagent was obtained from Bio-Rad (Hercules, CA). [γ-32P]ATP (3,000 Ci/mmol) and the enhanced chemiluminescence Western blot kit were obtained from Amersham (Arlington Heights, IL). Other reagents used were as described previously (4).

Animals. Rats were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility under a standard 12:12-h light-dark cycle and fed ad libitum. Between the hours of 7 AM and 1 PM, 70% PH was performed as previously described (3). At the indicated time points, the remnant liver tissue was harvested, flash-frozen in liquid nitrogen, and stored at −80°C. At least three animals were analyzed independently at each time point in the experiments shown, and representative samples were used. All studies were performed in accordance with the National Institutes of Health guidelines on the use of experimental animals, and approval had been obtained from the Animal Care, Use, and Research Studies Committee of the Minneapolis Medical Research Foundation.

Protein isolation. Liver tissue was homogenized in a 0.1% Tween 20 buffer containing protease and phosphatase inhibitors as previously described (4, 32). Nuclear and cytoplasmic extracts were obtained using a modification of previously described methods (56). Liver was homogenized in nuclear isolation buffer A (in mM: 25 Tris 7.5, 50 KCl, 2 MgCl2, and 1 EDTA) containing the protease and phosphatase inhibitors. Homogenates were centrifuged at 5,000 rpm for 10 min, and supernatant containing the cytoplasmic fraction was removed. The pellet containing nuclear protein was washed with buffer A and resuspended in nuclear isolation buffer B (25 mM Tris, pH 7.5, 0.42 M NaCl, 1.5 mM MgCl2, and 25% sucrose) with protease and phosphatase inhibitors. The resuspended pellet was incubated on ice for 30 min to release nuclear protein, sonicated, and clarified by centrifugation at 15,000 g for 20 min. The nuclear protein was then dialyzed against the 0.1% Tween 20 buffer. Protein concentrations of each fraction were determined using the DC protein reagent kit as recommended by the manufacturer.

Western blot analysis, immunoprecipitation, and kinase assays. Western blot and immunoprecipitation/Western blot analysis were performed as previously described (3, 4). In the Western blot studies, 50 µg of protein per lane were used. For immunoprecipitation and kinase studies, 500 µg of homogenate were used. Immunoprecipitation and kinase assays using Rb-GST and histone H1 as substrates were performed as previously described (4, 32) using the antibodies and substrates indicated. Immunodepletion was accomplished by incubating liver extracts with protein A/G beads precoated with an antibody to cyclins D1 or D3 (or an irrelevant control antibody) three successive times as previously described (4).

Recombinant proteins. GST-conjugated recombinant Cdc25A, Cdc25B, Cdk2, cyclin D1, and Rb were produced in the BL21 E. coli strain, and protein was isolated using glutathione-Sepharose beads as recommended by the manufacturer. Histone-tagged cyclin A was produced in E. coli and isolated using nickel-agarose beads as described (41). Baculoviruses encoding cyclin E-GST and Cdk2 were cotransfected into subconfluent SF9 cells, and protein was isolated using glutathione-Sepharose beads, as previously described (43).

Kinase assays and binding studies involving recombinant proteins. The activity of the purified recombinant cyclin H/Cdk7 was confirmed in Fig. 4C by incubating recombinant cyclin A and Cdk2 (produced in bacteria) in the presence of
ATP, as described by Poon et al. (41), followed by Cdk2 immunoprecipitation and kinase assays using Rb-GST as substrate. For the CAK activation of cyclin D1 immunoprecipitates in Fig. 4 B, cyclin D1 was immunoprecipitated from cytoplasmic extract, followed by washing of beads. These were then incubated for 30 min at 30°C in kinase buffer with 250 ng of recombinant cyclin H/Cdk7 in the presence of 50 mM ATP, as previously described (23). The beads were then washed and subjected to kinase assays using Rb-GST as substrate. In Fig. 4 D, cyclin D1 was immunoprecipitated from cytoplasmic extract, and the beads were washed extensively in Cdc25 buffer (100 mM Tris·HCl, pH 8.2, 250 mM NaCl, 5 mM EDTA, and 1 mM DTT) and incubated for 30 min at 30°C with 1 µg of Cdc25A or Cdc25B, as described (47). This was followed by washing of the beads and kinase assays as above. In Fig. 5 C, ~100 ng of recombinant cyclin D1 from bacteria were added to 250 µg of the indicated liver homogenates in Tween 20 buffer (or buffer alone), and the samples were incubated at 30°C for 30 min. This was followed by the addition of cyclin E-GST/Cdk2 prepared from baculoviruses (~10 ng of cyclin E per sample), and the samples were again incubated for 30 min. Glutathione-Sepharose beads were then added for an additional 3 h, and the beads were washed and subjected to histone H1 kinase assays as previously described (4). In a parallel experiment, cyclin D1-GST was added to the same liver homogenates for 2 h, collected on glutathione-Sepharose beads, washed, and resuspended in SDS-PAGE sample buffer for Western blot analysis.

RESULTS

Expression of G1 regulatory proteins and activation of kinase activity in regenerating rat liver. To further examine the regulation of G1-associated cyclin/Cdk activity in regenerating rat liver, we utilized the well-established model of 70% PH in young male Sprague-Dawley rats. Numerous studies have indicated that a large population of hepatocytes progress through the cell cycle in a relatively synchronous manner after PH, with peak DNA synthesis occurring at 24 h (33, 53). In the current study, liver tissue was harvested from quiescent liver (0 h) and at time points corresponding to G1 phase (6 and 12 h), the G1/S interval (18 h), and S phase (24 h), as well as at 48 h. Liver samples were homogenized in a low-detergent buffer that permits isolation of active cyclin D1/Cdk4 complexes (32) followed by Western blot analysis and immunoprecipitation-kinase assays.

Previous studies have documented upregulation of cyclin and Cdk mRNA and protein after PH in rodents (2–4, 7, 10, 16, 22, 29, 30, 44, 57, 59, 62). Similar to these previous studies, Western blot analysis demonstrated that cyclins D3 (33 kDa), D1 (36 kDa), and A (52 kDa) were sequentially upregulated, beginning in G1 phase (Fig. 1). An antibody directed against rat cyclin E recognized three main isoforms of this protein ranging from ~50 to 55 kDa, probably representing alternatively spliced variants of this gene (35, 46), that were markedly upregulated at 18 h. Addition of a cyclin E blocking peptide before Western blot prevented visualization of these bands, suggesting that they represent genuine cyclin E isoforms (data not shown). As previously demonstrated (3, 4), Cdk2 (33 kDa) and Cdk4 (34 kDa) were constitutively expressed in the liver and were modestly upregulated after PH. The Cdk2 antibody recognized two forms of this protein in liver after PH, with the lower form probably representing the Thr-160-phosphorylated form of Cdk2 (42). As shown in prior studies (4, 16, 22), p21 was upregulated after PH in the rat, whereas p27 was expressed in quiescent liver and minimally induced. Two forms of p27 were recognized after PH, possibly due to phosphorylation of this protein (48). An antibody directed against the amino terminus of mouse p57 readily recognized this protein in quiescent rat liver, and the level of this protein did not vary after PH.

As previously documented in rat and mouse liver after PH (4, 7, 16, 22, 29, 30, 59), progression of the hepatocytes through the cell cycle corresponded to activation of kinases associated with cyclins D1 and A, as well as Cdk2 and Cdk4 (Fig. 2A), which was substantially greater than the kinase activity associated with control antibodies (Fig. 2B). In addition, we found activation of cyclin D3- and E-associated kinase activity, which had not been previously demonstrated in this model. Studies in cell culture systems suggest that p27, p107, and p130 can inhibit cyclin/Cdk kinase activity but can also form active kinase complexes with these proteins (6, 12, 15, 21, 52, 60). Immunoprecipitation of p27 from regenerating rat liver revealed an active Rb kinase that was upregulated within 12 h after PH.
Immunoprecipitation of p107 and p130 also demonstrated association with active Rb kinases that were upregulated at 24 h. These results suggest for the first time that p27, p107, and p130 are each components of active cyclin/Cdk holoenzymes in the regenerating liver.

Cyclin/Cdk expression and kinase activity in nuclear and cytoplasmic extracts. Studies in cell culture systems have suggested that the G1 cyclins function primarily in the nucleus (5, 50). Studies in regenerating liver, on the other hand, have suggested that cyclins A and B are present in cytoplasmic, nuclear, and membrane-containing fractions in quiescent and regenerating liver (30, 57). Furthermore, active cyclin A/Cdk2 kinase complexes have been shown to be present in the cytoplasm, nucleus, and microsomal and endocytic compartments in the liver after PH (7, 30, 59). Previous studies (3) and those of Loyer et al. (29) found detectable cyclin D1 expression in quiescent rat liver when using high-detergent homogenization buffer. The samples used in Figs. 1 and 2 were obtained in a low-detergent buffer and showed little detectable cyclin D1 expression in quiescent rat liver, consistent with the results of two other groups (16, 22). These results and the older findings in high-detergent buffer suggest that cyclin D1 may be present in membrane-containing fractions in quiescent rat liver. To further study the regulation of G1-associated cyclin/Cdk complexes, we prepared nuclear and cytoplasmic extracts from quiescent (0 h) and regenerating (24 h) liver. We chose to limit our analysis to the nucleus and cytoplasm because of studies in cell culture that indicated that the critical regulation of cyclin/Cdk complexes occurred in these fractions (13, 14, 31, 36).

Western blot analysis showed that cyclin D1 protein was upregulated in both the cytoplasmic and nuclear fractions after PH, although it was substantially more abundant in the cytoplasmic fraction (Fig. 3A). Similarly, cyclin D3 was upregulated in both compartments and was more abundant in the cytoplasm. Cyclin E isoforms were upregulated in both fractions, although the higher-molecular-weight form was present only in the nucleus. As previously demonstrated (7, 30), cyclin A was found in both the nucleus and cytoplasm after PH. Cdk2 was also found in both fractions, although the lower-molecular-weight species (representing the Thr-160-phosphorylated form) was more predominant in the nucleus. Cdk4 expression was barely detected in the cytoplasmic or nuclear fraction in quiescent liver and was upregulated in both fractions during liver regeneration. p21 was not detected in the nuclear or cytoplasmic fraction in quiescent liver and was upregulated in both fractions during liver regeneration. p27 was not detected in the nuclear or cytoplasmic fraction in quiescent liver and was upregulated in both compartments after PH. p27 was found in each fraction in both quiescent and regenerating liver. To confirm the efficacy of our cellular fractionation technique, we examined the expression of laminin B, which is an nuclear protein. As previously demonstrated (57), laminin B was present only in the nuclear fraction in both quiescent and regenerating liver.

Although cyclin D1 protein was found in higher abundance in the cytoplasm, cyclin D1-associated kinase activity was found primarily in the nuclear fraction (Fig. 3B). Similarly, cyclin E kinase activity was more abundant in the nucleus. Cdk2 activity, on the other hand, was found in both the nuclear and cytoplasmic fractions, consistent with previous results (7, 30). Cdk7 activity, which is constitutively present in regenerating liver (4), was detected only in the nuclear fraction, consistent with results in cell culture systems.

We further examined potential mechanisms for the disparity between cyclin D1 protein expression and associated kinase activity in the nuclear and cytoplasmic fractions after PH. One possibility is that cyclin D1 fails to form complexes with Cdk4 in the cytoplasm. To examine this, we immunoprecipitated cyclin D1 from each fraction and performed Western blot analysis for Cdk4 (Fig. 4A). This showed that cyclin D1/Cdk4
complexes were more abundant in the cytoplasm than in the nucleus, in contrast to the results for the kinase assays. This suggested that the cytoplasmic cyclin D1/Cdk4 complexes were present but inhibited. It is possible that an overabundance of Cip/Kip inhibitors in the cytoplasmic fraction could account for the inhibition of cyclin D1/Cdk4, although this was not evident by Western blot analysis (Fig. 3A). Furthermore, the abundance of cyclin D1/p27 complexes appeared to be proportional to the abundance of cyclin D1/Cdk4 complexes in the cytoplasmic and nuclear compartments (Fig. 4A), suggesting that p27 was not responsible for the relative inhibition of cytoplasmic kinase activity. Because of high background, we were unable to perform p21 Western blot analysis on cyclin D1 immunoprecipitates from rat liver. However, the expression of p21 was similar in the cytoplasmic and nuclear fraction by Western analysis (Fig. 3A).

The above data suggested that cytoplasmic cyclin D1/Cdk4 complexes may have been inhibited because they had not undergone the phosphorylation changes necessary for full activation. Cdk4 requires Thr-172 phosphorylation by CAK for full activation. To test whether the cytoplasmic cyclin D1/Cdk4 complexes could be activated by CAK, cyclin D1 immunoprecipitates from PH liver were subjected to immunoprecipitation with cyclin D1 (lanes 1, 2, 4, and 5) or a control antibody (lane 3). Beads containing immunoprecipitated proteins were washed and incubated with cyclin H/Cdk7 and ATP (lanes 2, 3, and 5) or ATP alone (lanes 1 and 4), as described in MATERIALS AND METHODS. Beads were then washed and subjected to kinase assays using Rb-GST as substrate. In lane 6, liver extract prepared in Tween 20 homogenization buffer was subjected to cyclin D1 ip and kinase assay without CAK activation. C: Cdk activation by recombinant CAK. Recombinant (rec) cyclin A and Cdk2 were mixed in the absence (lane 1) or presence (lane 2) of recombinant cyclin H/Cdk7, followed by immunoprecipitation of Cdk2 and kinase assay using Rb-GST as substrate. In lane 3, a control experiment without cyclin A/Cdk2 was performed. In lane 4, liver extract prepared in Tween 20 homogenization buffer underwent cyclin D1 immunoprecipitation and kinase assay without Cdc25 pretreatment.
shown in Fig. 4C, the recombinant cyclin H/Cdk7 activated significant Cdk2 kinase activity from these bacterially produced proteins. Because Cdk4 activity can also be regulated by Tyr-17 phosphorylation, we also tested whether a Cdc25 phosphatase could activate the cytoplasmic cyclin D1/Cdk4 complexes. As shown in Fig. 4D (lanes 2 and 3), recombinant Cdc25A and Cdc25B failed to induce kinase activity associated with cyclin D1 immunoprecipitates from regenerating liver. These experiments suggest that cytoplasmic cyclin D1/Cdk4 complexes present in the cytoplasmic fraction of the liver after PH are inactive because they have not undergone phosphorylation by Cdk.

Titration of p27 and Cdk-inhibitory activity by cyclin D1. Studies in cell culture systems suggest that cyclin D1 may regulate other cyclin/Cdk complexes by sequestering Cdk-inhibitory proteins such as p27 (51). According to this model, during G1 and early G2 phase, most p27 is monomeric and capable of inhibiting cyclin/Cdk complexes. As cyclin D1 is induced in G1 phase, it binds increasing amounts of p27, thereby preventing inhibition of cyclin E/Cdk2 and cyclin A/Cdk2 kinases acting later in the cell cycle. Our previous studies have shown that p27 accounts for the majority of the Cdk2-inhibitory activity found in extracts of quiescent mouse liver (4). Furthermore, as cyclin D1 is induced following PH, it binds increasing amounts of p27, suggesting that cyclin D1 could function to sequester p27 in the cytoplasm (4). To examine this issue further, we studied the functional association of cyclin D1 and p27 in quiescent (0 h) and S phase (24 h) liver extracts.

As shown in Fig. 5A, induction of cyclin D1 in rat liver after PH was associated with increased abundance of cyclin D1/p27 complexes, similar to the findings in regenerating mouse liver (4). To determine the proportion of p27 that was bound to cyclin D1 at these time points, cyclin D1 was immunodepleted from the extracts before Western blot analysis (Fig. 5A). This confirmed that little p27 was associated with cyclin D1 in 0-h liver. On the other hand, most p27 was removed by immunodepleting cyclin D1 from the 24-h extract, indicating that the majority of p27 was complexed with cyclin D1 at this time point. Cyclin D3/p27 complexes were present in 0-h liver, and the abundance of these increased only minimally after PH (Fig. 5B). Immunodepletion of cyclin D3 indicated that little p27 was associated with cyclin D3 at 0 or 24 h.

We surmised that most p27 in 0-h liver was monomeric and capable of inhibiting cyclin/Cdk complexes in quiescent liver, whereas at 24 h most p27 was sequestered by cyclin D1-containing complexes. To test this further, we used active, recombinant cyclin E/Cdk2 complexes to study the presence of Cdk inhibitors in these extracts and to determine whether recombinant cyclin D1 could titrate out these inhibitors. The cyclin E/Cdk2 complexes were added to 0- and 24-h liver extracts (or to buffer alone), followed by precipitation of these complexes for kinase assays (Fig. 5C). Some of the extracts were pretreated with recombinant cyclin D1 before the addition of cyclin E/Cdk2. When cyclin E/Cdk2 complexes were added to extract of 0-h liver, these complexes were inhibited, suggesting the presence of monomeric Cdk inhibitors. Pretreating the 0-h extract with cyclin D1 prevented inhibition of cyclin E/Cdk2, indicating that cyclin D1 could sequester the Cdk inhibitors. On the other hand, the cyclin E/Cdk2 complexes were not inhibited by the 24-h extract, suggesting that there were no monomeric inhibitors present during S phase. Pretreating the 24-h extract (or buffer) with cyclin D1 had no effect on the resulting cyclin E/Cdk2 activity, indicating that the recombinant cyclin D1 did not directly regulate the activity of the cyclin E/Cdk2 complexes or directly activate a histone H1 kinase. In a parallel experiment, recombinant cyclin D1 was added to the same liver homogenates, followed by collection of the protein with glutathione-Sepharose beads. The precipitated proteins were then subjected to Western blot analysis for p27. As shown in Fig. 5D, the recombinant cyclin D1 precipitated p27 from the 0-h extract but not the 24-h extract.

![Fig. 5. Regulation of p27 by cyclin D1. A: complex formation and immunodepletion of cyclin D1. Left: extracts of regenerating liver 0 and 24 h after PH were immunoprecipitated with an antibody to cyclin D1 and the precipitated proteins were subjected to Western blot analysis for p27. Right: extracts obtained at 0 and 24 h after PH were immunoprecipitated with antibody to cyclin D1 (lanes 2 and 4) or control IgG (lanes 1 and 3) as described in MATERIALS AND METHODS. Western blot analysis was then performed on the extracts as indicated. B: complex formation and immunodepletion of cyclin D3. Immunoprecipitation (left) and immunodepletion (right) followed by Western blot analysis was performed as indicated. C: cyclin E/Cdk2 inhibitory activity. Recombinant cyclin E/Cdk2 from baculoviruses was mixed with buffer (lanes 1–2) or extracts from liver 0 and 24 h after PH (lanes 3–4 and 5–6, respectively). Extracts in lanes 2, 4, and 6 had been pretreated with cyclin D1-GST before adding cyclin E/Cdk2, as described in MATERIALS AND METHODS. Recombinant cyclin E/Cdk2 complexes were then precipitated and used for histone H1 kinase assays. D: sequestration of p27 by cyclin D1. Cyclin D1-GST was added to extracts from liver 0 and 24 h after PH (lanes 2 and 4), followed by addition of glutathione-agarose beads (to all lanes). Lanes 1 and 3 show control. Beads were collected and washed, and the precipitated proteins were subjected to Western blot analysis for p27.](http://ajpgi.physiology.org/)
further suggests that monomeric p27 was present in quiescent liver (but not in S phase) and that this monomeric fraction could be sequestered by cyclin D1.

DISCUSSION

Although prior studies have demonstrated upregulation of cyclins and associated proteins in regenerating liver after PH, there is little information about the regulation of cyclin/Cdk complexes in this in vivo model of cell cycle progression. The present study offers new information regarding several aspects of cyclin/Cdk regulation in the liver. First, proteins that have been previously identified as Cdk inhibitors (p27, p107, and p130) are present in complexes containing active Rb kinases, underscoring the multifunctional nature of these proteins. Second, cyclin D1/Cdk4 activity is regulated by its subcellular localization, presumably via Thr-172 phosphorylation by cyclin H/Cdk7, suggesting another facet of kinase regulation that had not been examined in the regenerating liver. Finally, these studies offer further evidence that cyclin D1 may regulate other cyclin/Cdk complexes in the liver through its capacity to sequester Cdk inhibitors such as p27. These data underscore the complex nature of cyclin/Cdk interactions during liver regeneration.

After PH in young rats, a large number of hepatocytes progress through the cell cycle relatively synchronously, with peak DNA synthesis occurring at 24 h after the procedure. Progression of hepatocytes through the G1 phase cell cycle was associated with sequential upregulation of cyclins D3, D1, E, and A and activation of kinase activities associated with these proteins. Cdk2 and Cdk4 proteins are expressed constitutively in the liver (3, 4, 16, 22), and upregulation of these kinases parallels the expression of their respective cyclin partners. As previously demonstrated (4, 16, 22), p21 was upregulated after PH, whereas p27 was expressed in quiescent liver and minimally upregulated after PH. p57 was expressed in a pattern similar to p27, suggesting that these two proteins may play similar roles in the regenerating liver.

In vitro studies indicate that p27, p107, and p130 can inhibit Cdk activity (12, 34, 51, 58, 60). However, this effect appears to depend on the status of the associated cyclin/Cdk complex. For example, p27/cyclin A/Cdk2 complexes are inactive, whereas p27/cyclin D/Cdk4 complexes are active if there is only one p27 molecule per complex; at higher concentrations of p27, the cyclin D/Cdk4 complex is inhibited (6). Prior studies have suggested that p27-dependent Rb kinase activity is associated with D-type cyclins and does not vary during cell cycle progression (15, 52). In contrast, in the current study, p27-associated Rb kinase activity was not detected in quiescent liver but was induced after PH. The p27-associated kinase was upregulated earlier in G1 phase than the other kinases assayed in this study (beginning at 12 h), suggesting that it may play a role in early G1 phase. However, further study is necessary to examine the role and composition of active p27/cyclin/Cdk complexes in the liver.

p107 and p130 are capable of binding numerous cyclin/Cdk complexes as well as E2F proteins. p107 and p130 have been observed to inhibit cyclin/Cdk2 activity, but other studies suggest that the binding of p107 or p130 to cyclin A/Cdk2 may actually shift substrate specificity from histone H1 to Rb (12, 21, 60). Previous studies have demonstrated the appearance of phosphorylated forms of p107 and p130 in the liver after PH (18). In the current study, we detected Rb kinase activity associated with p107 and p130 that corresponded to S phase. These findings suggest that, in addition to playing a role in regulating E2F binding in the liver (18), p107 and p130 form active kinase complexes after PH.

Because cyclin D1 is implicated in the control of G1 phase progression in hepatocytes and other types of proliferating cells, we further examined the regulation of cyclin D1/Cdk4 complexes in regenerating rat liver. Studies in cell culture systems and in vitro data suggest that activation of the cyclin D1/Cdk4 complex is a multistep process. In quiescent cells, cyclin D1 is expressed at low levels and is usually the first cyclin to be induced by growth factors, whereas Cdk4 is constitutively expressed (49, 50). Expression of cyclin D1 and Cdk4 by transfection does not necessarily lead to efficient formation of cyclin D1/Cdk4 complexes, suggesting that growth factor-dependent assembly factors are required (9, 24, 26, 32). Prior studies in mitogen-stimulated cells suggest that cyclin D1/Cdk4 complex formation in the cytoplasm is followed by transportation into the nucleus and subsequent activation by CAK (13, 14). The factors responsible for cyclin D1/Cdk4 complex formation and nuclear transport have not been clearly established and may vary between cell types.

To study the potential role of complex assembly and nuclear importation of cyclin D1/Cdk4, we examined the expression and activity of these complexes in cytoplasmic and nuclear extracts from regenerating liver. Previous studies have demonstrated that cyclins and Cdns are distributed through diffuse intracellular locations in the regenerating liver. For example, cyclin B protein is present in the cytoplasm, nucleus, and membrane-containing fractions in both quiescent and regenerating liver (57). Active cyclin A/Cdk2 complexes can be found in the nuclear, cytoplasmic, microsomal, nuclear membrane, and endocytic compartments in the liver after PH (7, 29, 59). Consistent with these prior findings, we found that Cdk2 activity was present in both the cytoplasmic and nuclear fractions after PH. On the other hand, cyclin D1/Cdk4 kinase activity was found primarily in the nuclear fraction, even though cyclin D1/Cdk4 complexes were more abundant in the cytoplasm. This is similar to a recent study in T cells demonstrating that, although more cyclin D3/Cdk6 was found in the cytoplasm, the kinase activity associated with these complexes was detected only in the nucleus (31). The current findings suggest that cyclin A/Cdk2 and cyclin D1/Cdk4 may play distinct roles in different cellular compartments. Furthermore, the results demonstrate that cyclin D1-associated kinase activity in
the liver is significantly influenced by its subcellular location, in contrast to Cdk2 activity.

The relative inhibition of cytoplasmic cyclin D1/Cdk4 complexes in the liver could be due to several factors. First, there could be an overabundance of Cdk inhibitors in the cytoplasmic fraction after PH. However, this is unlikely to be the case, since Cdk2 activity was present in this fraction and Cdk2 is inhibited at lower concentrations of Cip/Kip inhibitors than is Cdk4 (6). When recombinant cyclin E/Cdk2 complexes were mixed with cytoplasmic extract from rat liver after PH, these complexes were not inhibited (data not shown), which also suggests that there was not an excess of monomeric Cdk inhibitors in this fraction. Furthermore, the concentrations of p21 and p27 were similar in the cytoplasmic and nuclear fractions. It is also unlikely that Ink4 proteins account for inhibition of the cytoplasmic cyclin D1/Cdk4 complexes, since these inhibitors act by preventing formation of cyclin D/Cdk4 complexes (51). Another possibility is that the cytoplasmic Cdk4 had not undergone phosphorylation changes that promote its activation. The activity of Cdk4 is regulated by two phosphorylation sites. Tyr-17 phosphorylation is inhibitory, and this can be reversed by the Cdc25 family of phosphatases (34, 55). In the current study, cytoplasmic cyclin D1/Cdk4 complexes were not activated by recombinant Cdc25, suggesting that Tyr-17 phosphorylation did not account for the kinase inhibition. The other critical Cdk4 phosphorylation site is Thr-172 (24, 34). Phosphorylation of this residue by cyclin H/Cdk7 is necessary for full kinase activation. This is thought to occur after cyclin D1/Cdk4 complex assembly, since cyclin H/Cdk7 does not phosphorylate monomeric Cdk4 efficiently (14, 24). In the current study, cytoplasmic cyclin D1/Cdk4 complexes from regenerating liver were activated by recombinant cyclin H/Cdk7. This suggests that the cytoplasmic cyclin D1/Cdk4 complexes were inhibited because they had not been phosphorylated at Thr-172 by cyclin H/Cdk7. Because Cdk7 activity was found only in the nuclear fraction in the liver (as in other cell types (45)), it is likely that cyclin D1/Cdk4 complexes must first be imported into the nucleus for kinase activation.

Studies in cell culture systems have suggested that activation of cyclin D1/Cdk4 requires nuclear importation. Neither cyclin D1 nor Cdk4 possesses a typical nuclear localization signal, so it has been assumed that other proteins act as chaperones (14, 26). However, the identity of these potential chaperones remains unclear. Candidates include Cdc37, calmodulin, heat shock protein 90, p21, and p27 (11, 26, 27, 54). As noted above, in vitro studies suggest that low concentrations of p21 and p27 promote assembly of active cyclin/Cdk complexes, whereas higher concentrations are inhibitory. Furthermore, p21 contains a nuclear localization signal and therefore is a candidate nuclear chaperone for cyclin D1/Cdk4 complexes. In Saos-2 cells, most cyclin D1-dependent kinase activity was associated with p21, and transfection experiments indicated that p21 can promote nuclear localization of cyclin D1/Cdk4 (26). Transfection of 3T3 cells with p21 promoted nuclear importation of a cyclin D1 mutant (T156A) that otherwise localized to the cytoplasm (14); however, the p21-transfected cells were growth arrested, consistent with numerous other transfection studies indicating that p21 inhibits cell cycle progression (38, 51).

The available data suggest that p21 plays a growth-inhibitory role in the regenerating liver (4, 56, 61) and is not required for nuclear importation and CAK activation of cyclin D1/Cdk4. Our prior studies indicate that hepatocytes progressed more rapidly through G1 phase after PH in p21−/− mice than in p21+/+ mice (4). Furthermore, cyclin D1-associated kinase activation occurred earlier and was more intense in the p21−/− mice. Therefore, p21 is clearly not necessary for cyclin D1/Cdk4 kinase activation. It is possible that p21 promotes cyclin D1/Cdk4 complex assembly and nuclear importation in normal liver, but other proteins assume this role in p21−/− mice. It is unlikely that binding of cyclin D1/Cdk4 to p27 is responsible for its transportation into the nucleus, since the abundance of cyclin D1/p27 complexes was proportional to that of cyclin D1 in the nuclear and cytoplasmic fractions. A recent study in fibroblasts suggests that either p21 or p27 could fulfill the role of assembly factor and nuclear chaperone of cyclin D1/Cdk4, a possibility that requires further testing in the regenerating liver (8). Determining the factors responsible for intracellular trafficking and CAK phosphorylation of cyclin D1/Cdk4 in the liver will require further study. The current results do suggest that cyclin D1/Cdk4 activity is significantly dependent on its intracellular location, providing another potential pathway by which this complex is regulated in the regenerating liver.

In cell culture systems, cyclin E also plays an essential role in the transition from G1 into S phase, but little is known about the regulation of this protein in the regenerating liver. In the current study, we found that three isoforms of cyclin E were dramatically upregulated at 18 h after PH, which corresponded to induction of cyclin E-associated kinase activity. The kinase activity associated with cyclin E was found predominately in the nuclear rather than the cytoplasmic fraction. Western blot analysis of cyclin E in these fractions revealed that the lower two isoforms of cyclin E were induced after PH in the cytoplasm, but the larger cyclin E isoform was found only in the nucleus after PH. This suggests the 55-kDa form of cyclin E may be the form that is present in active cyclin E/Cdk2 complexes. Prior studies have indicated that the shorter versions of cyclin E may be splice variants, at least one of which is incapable of binding Cdk2 (35, 46). Alternatively, cyclin E may be subject to autophosphorylation by cyclin E/Cdk2 complexes (25), and the 55-kDa form may represent a phosphorylated species in the nuclear extracts. The marked upregulation of cyclin E seen in this study contrasts with prior results (29) and suggests that induction of cyclin E/Cdk2 kinase activity plays a role in the transition of hepatocytes into S phase, as would be predicted by work in other types of cells in culture.
As cyclin D1 is induced during G1 phase, it progresses and other G1-regulatory proteins interact in the regeneration of liver. The current study offers further insight into the mechanisms by which cyclin D1 plays an important role in the regulation of hepatocyte proliferation. The current study offers further insight into the mechanisms by which cyclin D1 plays an important role in the regulation of liver regeneration. Hepatic cyclin D1/Cdk4 kinase activity appears to depend on permissive phosphorylation by cyclin H/Cdk7. This phosphorylation step is likely to require nuclear importation of cyclin D1/Cdk4, although the factor(s) responsible for this translocation remains to be identified. Furthermore, the current data further suggest that p27 plays a complex role in the regenerating liver. Although p27 appeared to be present in active cyclin/Cdk holoenzymes that were upregulated after PH, it also may play a role in downregulating Cdk2-associated kinase activity in the liver. Further studies using knockout mice or transfection reagents targeting specific regulatory proteins will be necessary to more clearly identify the mechanisms that control G1 cyclin/Cdk complexes in the regenerating liver.

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