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Regulation of Cyclin D-Dependent Kinase Activity in Rat Liver Regeneration

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The regulation of cyclin D-dependent kinase activity in tissue regeneration *in vivo* has not been fully described. In young adult rat liver after 70% partial hepatectomy, the association of cyclin D1 with cdk4 was significantly promoted during G1 phase and was maximal at 18 hr, corresponding mainly to late G1. Cyclin D1-dependent kinase activity also strongly increased during G1 phase. The timing of the induction of cyclin D1 / cdk4 complex assembly correlated well with that of cyclin D1-dependent kinase activity. At 18 hr after partial hepatectomy, the amounts of CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ were also maximal, while only one-tenth of $p21^{CIP1}$ and of $p27^{KIP1}$ was associated with cyclin D1. These findings suggest that cyclin D1, cdk4 and their association act as promoting factors, and that both $p21^{CIP1}$ and $p27^{KIP1}$ may have physiological functions as adaptor proteins in additions to their roles as CDK inhibitors in rat liver regeneration.

The progression and transitions of the eukaryotic cell cycle are governed by sequential activation of protein kinase complexes composed of a regulatory subunit, the cyclin, and its catalytic subunit, the cyclin-dependent kinase (cdk) (1). In mammalian cells, D-type cyclins (D1, D2, and D3) whose expressions depend on mitogenic stimulation form enzymatically active complexes with cdk4 and cdk6 (2, 3). These complexes catalyze phosphorylation of the retinoblastoma protein (pRb) (3, 4) whose phosphorylation is required for entry into the S phase. Cdk-inhibitory proteins (CKIs), induced by various antimitotic signals, can negatively regulate G1 phase progression. Two families of CKIs have been identified in mammalian cells on the basis of homology. The INK4 family, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, selectively inhibit cdk4 and cdk6 by binding to the cdk subunit alone. The second class of inhibitors, the CIP/KIP family, includes $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, and can inhibit a broad range of cyclin-cdk complexes (5). Alternatively, all CIP/KIP family members might act as adaptor proteins, since they promote the association of D-type cyclins with cdk4 (6).

The regulation of cyclin D-dependent kinase activity has been studied mainly in immortalized cells in which oncogenic processes might exert their greatest effect on regulators of G1 phase progression (7). The purpose of this study was to examine the regulation of cyclin D-dependent kinase activity *in vivo*. Liver regeneration after 70% partial hepatectomy (PH) in rats is a well-characterized experimental system for the study of cell cycle regulation (8). During the first 24 to 36 hours after PH, a significant population of hepatocytes progresses through the cell cycle in a synchronous manner, and peak DNA synthesis occurs at about 24 hours. Although there have been published studies in which the kinetics of cyclin D1 mRNA and protein expression have been demonstrated (9, 10, 11), the activity of cyclin D1-cdk4 complexes was unclear. Moreover, distinct patterns of cyclin D1 expression have been reported. To gain insight into the role of cyclin D1 in regenerating rat liver, we examined cyclin D1-dependent kinase activity. In addition, we investigated the expression of $p21^{CIP1}$ and $P27^{KIP1}$ and their association with cyclin D1 to examine their physiological roles in the regulation of cyclin D1-dependent kinase activity *in vivo*.

MATERIALS AND METHODS

Animal procedures. Five-week-old male F344 rats were subjected to 70% PH (12) and the remnant livers were harvested at the indicated timepoints after surgery. For BrdU labeling, rats were injected with 5-bromo-2′-deoxyuridine (BrdU, Sigma Chemical Co, 50 mg / kg body weight) and were sacrificed two hours later.

Immunohistochemical staining. Paraffin sections of liver tissue were deparaffinized, incubated in 2N HCl for 30 min, washed several

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times with phosphate-buffered saline, and stained with anti-BrdU antibody (Amersham International Plc). The procedure was performed as indicated in Vectastain ABC Kits (Vector Laboratories).

Northern analysis. Total RNA was isolated from liver tissues using the guanidium thiocyanate-cesium chloride centrifugation method (13) and subjected to Northern analysis as described (14). The cDNA probe used was an EcoRI fragment of mouse cyclin D1 (15).

Immunoprecipitation–Western (IP-Western) analysis. Liver tissue was homogenized in Nonidet P-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodiumdeoxycholate) containing 5 μg/ml of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaF, 10 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate. IP-Western assay was performed as described (16). Protein concentration was estimated using the DC protein assay (Bio-Rad). For IP-Western analysis of cdk4, we used a rabbit polyclonal antibody and mouse monoclonal antibody (mAb) as previously reported (16). For cyclin D1, mouse mAb specific for mouse cyclin D1 was used including pRb kinase assay, and was a gift of C. J. Sherr. The following antibodies were purchased commercially, for $p21^{CIP1}$ immunoprecipitations and Western blots, SC-397 (Santa Cruz Biotechnology, Inc.) and Ab-4 (Oncogene Research Products), respectively, and for $p27^{KIP1}$ immunoprecipitations and Western blots, SC-528 (Santa Cruz) and K25020 mAb (Transduction Laboratories), respectively.

Immune complex kinase assay. The assay for pRb kinase activity was performed as previously described (4). In brief, liver tissue was homogenized in Tween 20 lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol) containing protease and phosphatase inhibitors. After immunoprecipitation with cyclin D1 mAb, cyclin D1-cdk4 complexes recovered on protein G-Sepharose (Pharmacia Biotech) were incubated for 30 min at 30°C in 20 μl of kinase buffer (50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM NaF, 0.1 mM sodium orthovanadate) supplemented with 20 μM ATP, 10 μCi of [γ-^{32}p]ATP (Amersham), and 0.5 μg of purified Glutathion S-transferase (GST)-pRb substrate. The samples were boiled in sample buffer and separated by SDS-PAGE.

RESULTS AND DISCUSSION

Cell cycle progression following PH in the rat. After 70% PH in young adult rats, as many as 95% of hepatocytes undergo at least a single cycle of replication in restoring the original size of the liver (8), and the exact timing of DNA synthesis after PH varies with the age of the rat (17). To examine the DNA synthesis profile of young adult rats, we used immunohistochemical staining of liver sections of BrdU-injected rats with anti-BrdU antibody. Labeled nuclei were first evident in the periportal regions at 18 hr, and then proceeded to the pericentral areas. Although the distribution of labeled hepatocytes in lobules was heterogeneous at 18 hr, the peaks of labeling occurred at 24 hr with good synchrony (Fig. 1), indicating that a large number of hepatocytes reached the G1/S boundary approximately 21 hr after PH. In all BrdU-injected rats, BrdU incorporation into gut epithelial cells was normal (data not shown), providing a control for BrdU injection and staining.

The expression of cyclin D1 and cdk4 in regenerating rat liver. Expression of the 4.2 kb cyclin D1 mRNA appeared as a faint band in normal rat liver. There was a significant induction after 8 hr post-PH, and it reached a maximum at 15 hr (Fig. 2). To study the correlation between mRNA and the corresponding protein, we examined the level of the protein using IP-

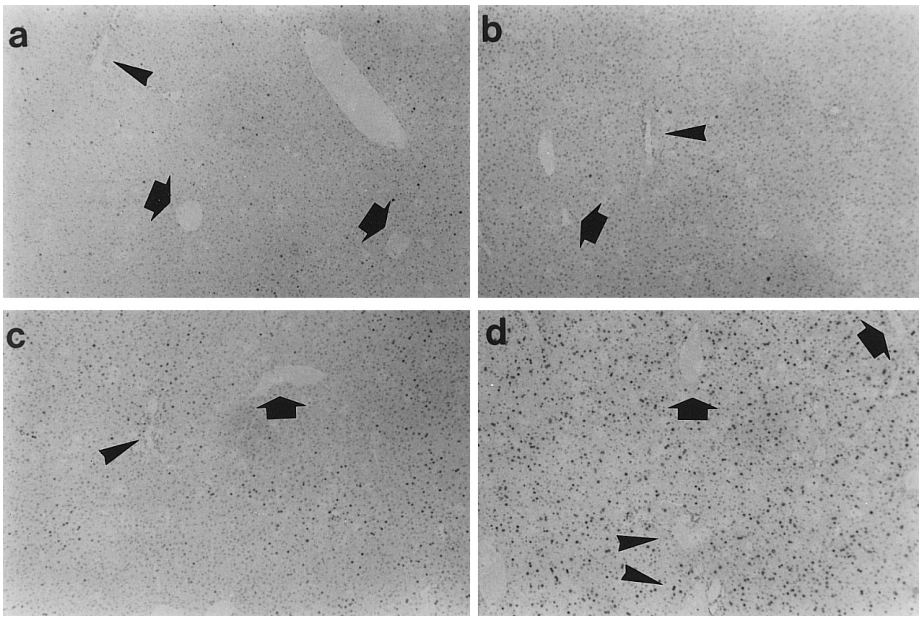

FIG. 1. Immunohistochemical staining of liver sections using anti-BrdU antibody after PH. (a) Control rat, 0 hr, (b) 12 hr post-PH, (c) 18 hr post-PH, (d) 24 hr post-PH. Dark nuclear staining reveals the presence of BrdU in newly synthesized DNA. Arrowheads and arrows indicate the portal triad and central vein, respectively. Magnification in all panels, X20.

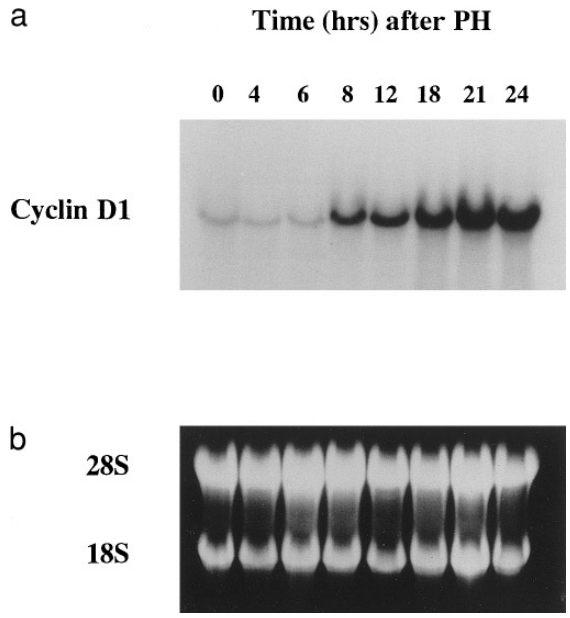

FIG. 2. Expression of cyclin D1 mRNA in regenerating rat liver. (a) Total RNA (25 μg / lane) from regenerating liver at the indicated timepoints were isolated, and subjected to Northern analysis using a cDNA probe to mouse cyclin D1. (b) Ethidium bromide-stained 28S and 18S ribosomal bands are shown as a control for total amounts of RNAs.

Western analysis throughout the 24-h detailed kinetic synchrony. Cyclin D1 was detected at a low level in quiescent liver, but drastically increased in amount between 8 and 15 hr post-PH, and then remained at a high level for the remaining period of study (Fig. 3a). These findings are comparable to those obtained from Northern blot analysis. Although a similar induction of cyclin D1 in male F344 regenerating rat liver has been reported (11), the distinct pattern of its protein expression was previously reported. In Sprague-Dawley rats, little change in cyclin D1 protein level was detected following PH (9), whereas in culture, this level strongly increased in mitogen-stimulated hepatocytes (18). The discrepancy between these findings may be the result from use of different strains or systems (*in vivo* compared with *in vitro*). Cdk4 protein was also detected in normal rat liver. Although cdk4 gradually increased throughout G1 after PH, it did not vary significantly in amount compared with cyclin D1 (Fig. 3a).

Induction of cyclin D1-dependent kinase activity. Formation of active holoenzymes of D-type cyclins and cdks governs the rate of progression of mammalian cells through the G1 phase of the cell cycle by phosphorylation of the retinoblastoma protein (1). To estimate the rate of formation of cyclin D1-cdk4 complexes, we examined kinase complex assembly by detection of cyclin D1 by Western blot after immunoprecipitation of cdk4 (Fig. 3a). In samples of 0 hr and 4 hr livers, complex formation could not be sufficiently visualized, but greatly increased thereafter and reached a maximum at 18 hr. There is no report describing the kinetic expression of cyclin D-dependent kinase activity in either cultured hepatocytes or regenerating liver after PH. To determine whether cyclin D1-cdk4 complexes were active, we further analyzed cyclin D1-dependent kinase activity using pRb as a substrate. As shown in Figure 3b, it was very low in quiescent liver, but its level significantly increased through G1 phase and reached a maximum at 18 hr, corresponding mainly to late G1. The activation of cyclin D1-dependent kinase, the association of cyclin D1 with cdk4, and the expression of cyclin D1 protein all revealed a similar pattern of induction (Fig. 3a, 3b). These findings indicate that, in regenerating rat liver, cyclin D1 accumulation facilitates the progressive assembly of enzymatically active cyclin D1-cdk4 complexes as liver cells progress through G1 phase.

Expression of CIP/KIP family members in regenerating rat liver. CKIs play an important role in regulating cdk activity. To study the regulation of cyclin D1-dependent kinase activity further, we examined the expression of $p21^{CIP1}$ and $p27^{KIP1}$. The expression of $p21^{CIP1}$ was barely detectable in normal rat liver. A small increase was noted by 4 hr after PH, while after 18 hr there was more than a 5-fold increase in abundance of this protein. By contrast, $p27^{KIP1}$ was constantly expressed at high levels throughout G1, but its expression gradually decreased from 21 hr, near the G1/S boundary (Fig. 3c). Our findings of a decrease in $p27^{KIP1}$ protein only after G1/S transition are consistent with recent findings which demonstrated that cyclin E-cdk2 catalytic activity induced from late G1 is required to down-regulate $p27^{KIP1}$ protein levels (19). As liver cells progressed toward S phase, the assembly of enzymatically active cyclin D1-cdk4 complexes was induced and cyclin D1-dependent kinase activity drastically increased. This increase was maximal at 18 hr in late G1 phase (Fig. 3b). Interestingly, the amounts of both $p21^{CIP1}$ and $p27^{KIP1}$ were near maximal at the same time. One interpretation of these findings is that the amount of cyclin D1-cdk4 complexes accumulating in G1 overrides inhibitory activity. If this were true, cyclin D1-dependent kinase activity could first make an appearance once the amount of assembled cyclin D1-cdk4 complexes was high enough to exceed inhibitory activity. Thus, in tissue extracts from regenerating liver, there should be a threshold level of cyclin D1-cdk4 complexes required to override inhibitory activity. However, cyclin D1-dependent kinase activity already increased from 6 hr post-PH, when a low level of cyclin D1-cdk4 complexes was present. Indeed, we observed no threshold level. Moreover, as shown here, a strong increase in kinase complex assembly closely paralleled a significant induction of cyclin D1-dependent kinase activity. Our findings thus do not support the above

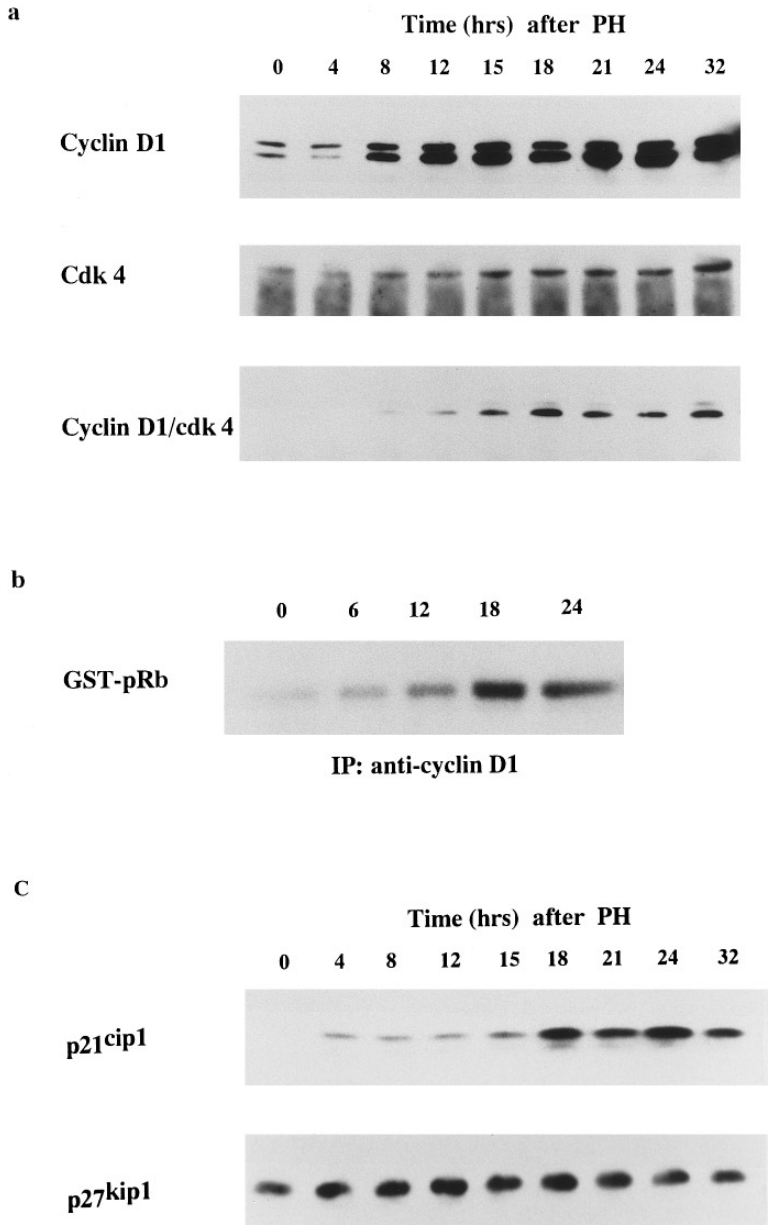

FIG. 3. Cyclin D1-associated kinase activity depends on cyclin D1/cdk4 complex formation. (a and c) The protein levels of cyclin D1, cdk4, $p21^{CIP1}$, and $p27^{KIP1}$ in regenerative rat liver (7 mg of protein / lane) were examined by immunoprecipitation and subsequent Western blotting. To detect cyclin D1 associated with cdk4, liver extracts were immunoprecipitated with antibody to cdk4 and subjected to Western blotting with antibody to cyclin D1. (b) Liver extracts (7 mg of protein / lane) were immunoprecipitated with antibody to cyclin D1 and assayed for pRb kinase activity.

explanation. Alternatively, $p21^{CIP1}$ and $p27^{KIP1}$ may not function as CDK inhibitors in regenerating liver. Recently, in addition to functioning as inhibitors of active cyclin D-cdk4 complexes, both inhibitors have been reported to play roles as adaptor proteins that promote the assembly of active kinase complexes and stabilize them. At high concentrations, they inhibit active kinase complexes. A recent study has demonstrated that, in transgenic mice with forced overexpression of $p21^{CIP1}$ specifically in hepatocytes, the transgenic $p21^{CIP1}$ protein was associated with most of the cyclin D-cdk4 complexes in liver, and hepatocyte proliferation was severely impaired in regenerating liver after PH (20). Conversely, at low concentrations, $p21^{CIP1}$ promotes the activity of cyclin D-cdk4 complexes, and $p27^{KIP1}$ does not inhibit it (6). This raises the possibility that CIP/KIP family members act as adaptor proteins. When we immunoprecipitated cyclin D1 and measured associated $p21^{CIP1}$ in 18-hr regenerative rat liver in which cyclin D1-dependent kinase activity, $p21^{CIP1}$,

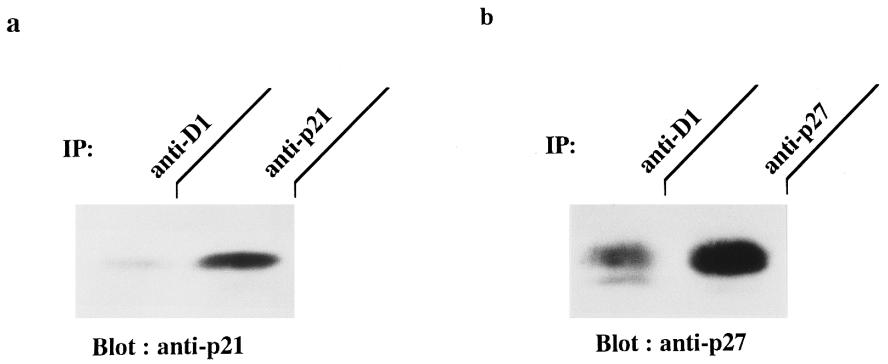

FIG. 4. Association of $p21^{CIP1}$ or $p27^{KIP1}$ with cyclin D1 in 18-h regenerating liver. Liver extracts (5 mg of protein / lane) at 18 hr after 70% PH were immunoprecipitated with antibody to cyclin D1 (anti-D1), to $p21^{CIP1}$ (anti-p21), or to $p27^{KIP1}$ (anti-p27) followed by Western blotting for $p21^{CIP1}$ (left) and $p27^{KIP1}$ (right).

and $p27^{KIP1}$ all reached near maximal levels, only one-tenth of $p21^{CIP1}$ was associated with cyclin D1 (Fig. 4a). We also observed a similar low ratio of $p27^{KIP1}$ to cyclin D1 (Fig. 4b). These findings are consistent with previous results demonstrating that $p27^{KIP1}$ is sequestered in proliferating cells (21). Therefore, cyclin D1 may associate with $p27^{KIP1}$ at low concentrations. Taken together, our findings suggest that both $p21^{CIP1}$ and $p27^{KIP1}$ may physiologically regulate the activity of cyclin D1-cdk4 complexes by acting as adaptor proteins throughout G1 following PH.

In conclusion, drastic induction of cyclin D1-dependent kinase activity depends on the progressive assembly of enzymatically active cyclin D1-cdk4 complexes facilitated by strong increase in cyclin D1 mRNA and protein throughout the G1 phase in regenerative rat liver. Moreover, $p21^{CIP1}$ and $p27^{KIP1}$ may have physiological functions as adaptor proteins in addition to their roles as CDK inhibitors.

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