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Cdk4 Activation Is Dependent on the Subunit Rearrangement in the Complexes

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Although several factors have been implicated in the regulation of Cdk4 activity, little is known regarding the contributions of cyclin-dependent kinase inhibitors (CKIs) in Cdk4 activation in the mid G1 phase. Using a mouse macrophage cell line (Bac1.2F5), we found that most of Cdk4 bound to p15 when cells were in a quiescent state. Following CSF-1 stimulation, Cdk4 bound to cyclin D1 and then to p21, concomitant with the dissociation of p15 from the complexes. The activation of Cdk4 correlated well with p21 binding to the complexes, and the majority of active Cdk4 complexes contained p21. During regeneration of mouse liver after partial hepatectomy, Cdk4 activity coincided precisely with ternary complex formation of cyclin D1/Cdk4/p21. Using the baculovirus expression system, we succeeded in reconstituting a capacity for Cdk4 activation in insect cells, forming an active cyclin D1/Cdk4/p21 ternary complex. Taken together, it is suggested that p21 and cyclin D1 act cooperatively as activators of Cdk4 through the release of CKIs of the INK4 family.

Cyclin-dependent kinases (Cdks) play a key role in cell cycle progression in eukaryotic cells (1), and their activities are regulated through a number of mechanisms (2). Cdk activation involves both an association with a cyclin and phosphorylation of a conserved threonine residue by Cdk-activating kinase (CAK). In addition, individual Cdks are temporally activated by their cognate cyclins whose expression is induced in certain situations (3).

Another regulatory mechanism has emerged with the discovery of the Cdk inhibitors (Cyclin-dependent Kinase Inhibitors, CKIs) (4, 5). Two families of CKIs, the INK4 family and the Cip/Kip family, have been reported in mammalian cells (6). These two classes are structurally distinct, and their members interact with cyclins and Cdks in completely different ways. Members of the INK4 family (p15, p16, p18, and p19) share characteristic fourfold ankyrin repeats and bind specifically to Cdk4 and its close homolog Cdk6 (7–11). In contrast, Cip/Kip proteins (p21, p27, and p57) share a homologous amino-terminal domain that contains contiguous cyclin- and Cdk-binding regions, and bind to a wide range of cyclin/Cdk complexes (12–20).

Another interesting characteristic of the Cip/Kip family of CKIs is that the p21 molecule can be a component of catalytically active enzymes, raising the possibility that p21 may have roles to play in cell cycle regulation other than as a CKI (21, 22). Recently, it has been proposed that members of the Cip/Kip family of CKIs act as assembly factors of cyclin D1/Cdk4 (23). However, little is known regarding the role served by this new function in Cdk4 activation during G1 progression.

It has previously been reported that overexpression of cyclin D1 in quiescent cells does not lead to successful activation of Cdk4, suggesting that other factors which might be induced by mitogen stimulation are necessary for Cdk4 activation (24). Very recently, severe impairment of Cdk4 activation has been shown in embryonic fibroblasts derived from p21/p27 double knock mice, suggesting that p21 and p27 might be a Cdk4-activating factor (25).

In this study, we demonstrated that while the major portion of Cdk4 in quiescent murine macrophage cells

Abbreviations used: BrdU, bromodeoxyuridine; PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside.

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(Bac1.2F5) is associated in a complex with p15, most Cdk4 forms an active ternary complex in mid to late G1 with cyclin D1 and p21 from which p15 has been dissociated. Furthermore, p21 had a similar effect *in vivo* on the activation of Cdk4 as observed in a mouse model of liver regeneration. In *in vitro* experiments, p21 formed an active cyclin D1/Cdk4/p21 complexes through the dissociation of p15, further supporting the concept of a novel function for p21 as a facilitator of the release of members of the INK4 family from Cdk4.

MATERIALS AND METHODS

Antibodies. Antibodies to p21 and p15 were raised in rabbits by immunizing with the bacterially produced 58 C-terminal amino acid residues of hexahistidine-tagged murine p21 and p15, respectively. Monoclonal antibody to cyclin D1 was a generous gift from Dr. C. J. Sherr, and antibody to Cdk4 (H-22) was purchased from Santa Cruz Co. (Santa Cruz, CA).

Synchronization of Bac1.2F5 and cell cycle analysis. Murine macrophage cells (Bac1.2F5) were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 20% L-cell-conditioned medium as a source of CSF-1. Cells were synchronized in G_0 by depletion of CSF-1 for 18 h, and were then restimulated with CSF-1. For cell cycle analysis, cells were labeled with bromodeoxyuridine (BrdU) and fixed with 70% ethanol on ice for 30 min, denatured with 2 N HCl/0.5% Triton X-100 for 30 min at room temperature and neutralized with borax buffer (pH 8.5). Labeled cells were stained with FITC-conjugated antibody to BrdU (Becton–Dickinson) containing 5 μg/ml propidium iodide, and were submitted to analysis on a FACSCalibur flow cytometer. Data were analyzed using Cell Quest software (Becton–Dickinson). Approximately 80% of the cells entered S phase 14 h after CSF-1 stimulation.

Protein analysis. Synchronized Bac1.2F5 cells ($1–3 \times 10^6$) were lysed with 50 mM Tris (pH 8.0), 120 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.5% NP-40, 20 units/ml of aprotinin (Bayer), 0.1 μM PMSF, 1 mM NaF, 0.1 mM Na_3VO_4, and 10 mM β-glycerophosphate. Lysates were immunoprecipitated with specific antibodies and separated by SDS–PAGE followed by Western blotting. Protein signals were enhanced by chemiluminescence (Amersham).

Kinase assay. Kinase activity was determined at 30°C for 30 min in a 30-μl reaction mixture containing 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 10 μM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4, 0.1 μM PMSF, 10 μM ATP and 185 kBq of [γ-^{32}P]ATP (222 Tbq/mmol; NEN). GST–pRb (0.2 μg) was used as a substrate. The reaction products were separated on SDS–PAGE. Phosphorylated proteins were detected by autoradiography and quantitated using a Fuji Image Analyzer BAS-1500.

For measurement of kinase activity in mice during liver regeneration after partial hepatectomy, 0.3 g of regenerating liver was homogenized in 6 ml of IP-kinase buffer, and briefly sonicated and centrifuged at 27,000*g* for 10 min. An equal amount (7.5 mg) of proteins of clear supernatant was subjected to immunoprecipitation using antibody against cyclin D1, Cdk4 or p21. The immunoprecipitates obtained were used in the kinase assay described above.

Partial hepatectomy. Male BALB/c mice (8–10 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were allowed to acclimatize to our animal facility for at least one week under standard environmental conditions (23°C, a 12-h light/dark cycle, and food and water permitted *ad libitum*). A 70% partial hepatectomy was performed under ether anesthesia according to the method of Higgins and Anderson (26). A sham operation (laparotomy and liver manipulation without tissue removal) was performed as a control. All operations were performed between 8 A.M. and noon. At various times after surgery, mice were sacrificed and the regenerating liver was removed, rinsed in phosphate buffered saline, quickly frozen in liquid nitrogen, and stored at −140°C until required. The experimental protocols for this study were approved by the Committee for Animal Experiments of the National Institute for Longevity Sciences.

RNA preparation and Northern analysis. Total RNA was isolated from liver tissues using guanidinium thiocyanate, and cesium trifluoroacetate (CsTFA) centrifugation (27). Briefly, frozen tissues were homogenized in 5.5 M guanidine thiocyanate, layered over CsTFA (Pharmacia Biotech), and centrifuged at 85,000*g* for 24 h at 15°C. The pellet was resuspended in 4 M guanidine thiocyanate (Fluka) and precipitated in ethanol containing acetic acid. The obtained pellet was resuspended in TE and precipitated in ethanol containing NaCl. The final RNA pellet was dissolved in DEPC-treated H_2O and stored at −80°C until use. Total RNA (20 μg/lane) was subjected to electrophoresis on a 1% formaldehyde-agarose gel. Hybridization was carried out in 50% formamide at 42°C for 24 h.

Metabolic labeling and immunoprecipitation using insect cells. Spodoptera frugiperda (Sf9) cells (1×10^6 cells) were infected with recombinant baculoviruses encoding cyclin D1, Cdk4, p21, and p15 at a multiplicity of infection (m.o.i.) of 3 (for Cdk4) or 10 (for cyclin D1, p21, and p15). Forty-eight hours after infection, cells were labeled for 4 h with 0.1 mCi/ml of [^{35}S]methionine (1000 Ci/mmol; ICN, Irvine, CA) and lysed in 500 ml of IP-kinase buffer. The lysates were clarified by centrifugation and incubated with the specific antibodies for 1 h at 4°C. The resultant lysates were further incubated with protein A–Sepharose beads for 1 h at 4°C. Immunocomplexes were collected by centrifugation, washed four times in IP-kinase buffer, and divided into two portions; one for SDS–PAGE and the other for the kinase assay.

RESULTS AND DISCUSSION

Most Cdk4 is associated with p15 in quiescent cells. Although, using p21/p27 double knockout mice, it has recently been reported that p21 and p27 are essential for activation of Cdk4, the detail mechanism by which p21 or p27 regulate Cdk4 activity during G1 progression is largely unknown. To address this question, we first attempted to identify the factors that associate with Cdk4 during G1 progression. In Bac1.2F5, a murine macrophage cell line, the p16 gene is deleted, and p15 is expressed at its highest level among members of the INK4 family of CKIs (data not shown). It is also known that withdrawal of colony-stimulating factor 1 (CSF-1) leads to cell cycle arrest in early G1 (28).

Western analysis of the Cdk4 immunoprecipitates from CSF-1-depleted Bac1.2F5 cells revealed that Cdk4 bound to p15 when cells were in a quiescent state (Fig. 1A). When restimulated with CSF-1, Bac1.2F5 cells synchronously entered the cell cycle. Cdk4 had begun to bind cyclin D1 at 1 h after CSF-1 stimulation when the level of Cdk4-bound p15 had not decreased (Fig. 1A, upper panel). Four hours after CSF-1 stimulation, p21 was detected in the Cdk4 immunoprecipitates (Fig. 1A, third panel), and p15 had concomitantly disappeared from the complex, despite the fact that the expression of p15 protein persisted during G1 progres-

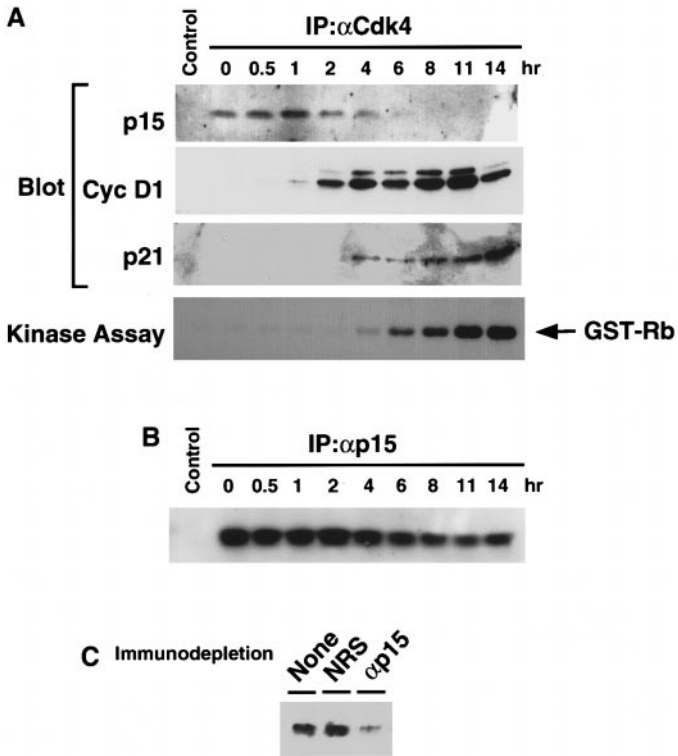

FIG. 1. Cdk4 activation coincides with the association of this molecule with cyclin D1 and p21 and with the dissociation of p15. Bac1,2F5 cells, a macrophage cell line, were synchronized by CSF-1 depletion for 18 h, and were then stimulated with CSF-1 and lysed at the indicated times. (A) Whole-cell extracts (from 2×10^6 cells) were immunoprecipitated using anti-Cdk4 antibody. The immunoprecipitates were then subjected to Western blotting with antibody against p15 (upper panel), cyclin D1 (second panel) or p21 (third panel), or to kinase assay using GST-Rb as a substrate (bottom panel). (B) Lysates (from 2×10^6 cells) were immunoprecipitated with antibody against p15, and the immunoprecipitates were subjected to Western blotting with the same anti-p15 antibody. (C) Extracts from quiescent cells (70 μg) were precleared four times using either normal rabbit serum (NRS) or anti-p15 antibody, and the supernatants were subjected to Western blotting using anti-Cdk4 antibody.

sion (Fig. 1B). The amount of Cdk4 was not varied during this experimental periods (data not shown). Although cyclin D1 was readily detected in the Cdk4 immunoprecipitate at 2 h after stimulation (Fig. 1A, second panel), almost no Cdk4 kinase activity was observed at this time (Fig. 1A, bottom panel). However, kinase activity was readily detected at 4–6 h after CSF-1 stimulation, suggesting that association with both cyclin D1 and p21 is required for full activation of Cdk4.

To determine the proportion of Cdk4 molecules that associated with p15 in quiescent cells, we immunodepleted Bac1.2F5 cell extracts by means of four rounds of immunoprecipitation with the antibody against p15, and examined the supernatant for the presence of remaining Cdk4 by Western blotting. As shown in Fig. 1C, the amount of Cdk4 was substantially reduced after the fourth immunoprecipitation with anti-p15 antibody, whereas there was no Cdk4 depletion when normal rabbit serum (NRS) was used, suggesting that under quiescent conditions, most Cdk4 is associated with p15. Thus, p15 appears to function in preventing Cdk4 from premature activation in the absence of mitogen stimulation.

p21 forms an active complex with cyclin D1/Cdk4 during G1 progression. To further substantiate the concept that p21 participates in Cdk4 activation during G1 progression, we examined whether p21 could form kinase-active complexes. As shown in Fig. 2A, the p21 immunoprecipitates of quiescent cells (G_0) exhibited almost no kinase activity. However, p21-associated kinase activity was readily detectable at 4–6 h after CSF-1 stimulation and increased gradually until 14 h (Fig. 2A, bottom panel). IP-Western blotting using the p21 immunoprecipitates revealed the ternary complex formation of cyclin D1/Cdk4/p21 at 4–6 h after CSF-1 stimulation (Fig. 2A), suggesting that the p21-associated kinase activity was attributable to the ternary complex of cyclin D1/Cdk4/p21.

Next, in order to determine the proportion of cyclin D1/Cdk4 complexes associated with p21, we depleted p21 from cell extracts by sequential immunoprecipitation. The anti-p21 antibody, but not normal rabbit serum (NRS), immunoprecipitated cyclin D1-bound Cdk4 (Fig. 2B). Incubation of cell lysates with anti-p21 antibody recovered the majority of Cdk4, and after 4 rounds of immunodepletion, no cyclin D1-bound Cdk4 was detected (αp21-4 in Fig. 2B). On the other hand, a similar experiment with NRS failed to deplete the lysates of cyclin D1-bound Cdk4 (NRS-4 in Fig. 2B). Taken together, these results indicate that the majority of cyclin D1/Cdk4 complexes are associated with p21 during G1 progression in Bac1.2F5 cells.

Since it has been reported that p21 also associates with active Cdk2 complexes in normal fibroblasts (21, 22), we next attempted to determine the proportion of p21-associated pRb kinase activity which could be attributed to the cyclin D1/Cdk4 complexes. The p21 immunoprecipitates from the Bac1.2F5 cell lysates contained a high level of pRb kinase activity (Fig. 2C). In contrast, immunodepletion by either anti-cyclin D1 or anti-Cdk4 antibody substantially reduced p21-associated kinase activity, suggesting that most cyclin D1/Cdk4 formed kinase-active complexes with p21.

p21 forms active kinase complexes with Cdk4 during liver regeneration in vivo. We further examined whether the association of p21 with active Cdk4 complexes contributes not only to cellular proliferation *in vitro* but to organ regrowth *in vivo* as well. Although hepatocytes in adult animals are terminally differentiated and mitotically quiescent, the liver is known to retain the ability to rapidly regenerate after partial resection (29). As shown in Fig. 3A, cyclin D1 mRNA

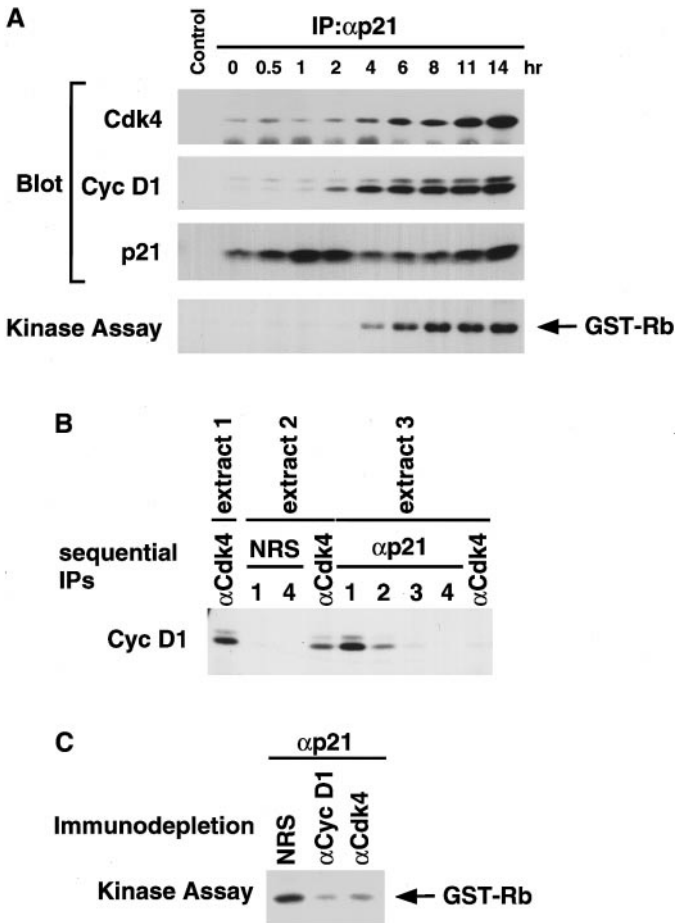

FIG. 2. The majority of kinase-active cyclin D1/Cdk4 complexes contain p21. Cell synchronization and stimulation by CSF-1 were performed under the same conditions as described in the legend to Fig. 1. The intervals (in hours) following stimulation are indicated at the top of A. (A) Lysates (from 2×10^6 cells) were immunoprecipitated with anti-p21 antibody, and the immunoprecipitates were subjected to Western blotting with antibodies against Cdk4 (upper panel), cyclin D1 (second panel) or p21 (third panel), or to kinase assay using GST-Rb as a substrate (bottom panel). (B) Cell extracts were divided into 3 portions, and extracts 2 and 3 were subjected to four successive rounds of immunoprecipitation using either normal rabbit serum (NRS) or anti-p21 antibody, respectively and extract 1 served as a control without immunoprecipitation with NRS or anti-p21 antibody. Extracts 1, 2, and 3 were then immunoprecipitated with anti-Cdk4 antibody and each immunoprecipitate was subjected to Western blotting with anti-cyclin D1 antibody. Numbers under NRS or αp21 indicate the specific round of immunoprecipitation. (C) Cell lysates at 6 h after CSF-1 stimulation were precleared twice with either protein A–Sepharose beads, anti-cyclin D1 antibody or anti-Cdk4 antibody. The resultant lysates were subjected to immunoprecipitation with anti-p21 antibody followed by the determination of p21-associated Rb kinase activity.

was barely detectable in resting mouse liver, but its level markedly increased at 48 h after partial hepatectomy (PH, 70%) when BrdU uptake also peaked (data not shown), and remained elevated for 120 h. Sham operation failed to induce cyclin D1 mRNA (data not shown).

Interestingly, the expression of p21 mRNA during liver regeneration was biphasic. The first peak was detected 6–12 h after PH as it was after the sham operation, suggesting that this early induction of p21 mRNA may be due to stress from the surgery and not to liver regeneration per se. The second peak of p21 mRNA was specifically observed at 48–72 h after PH, and had disappeared almost completely by 96 h (Fig. 3A). The second induction of p21 mRNA was not observed in the sham-operated animals, and was consid-

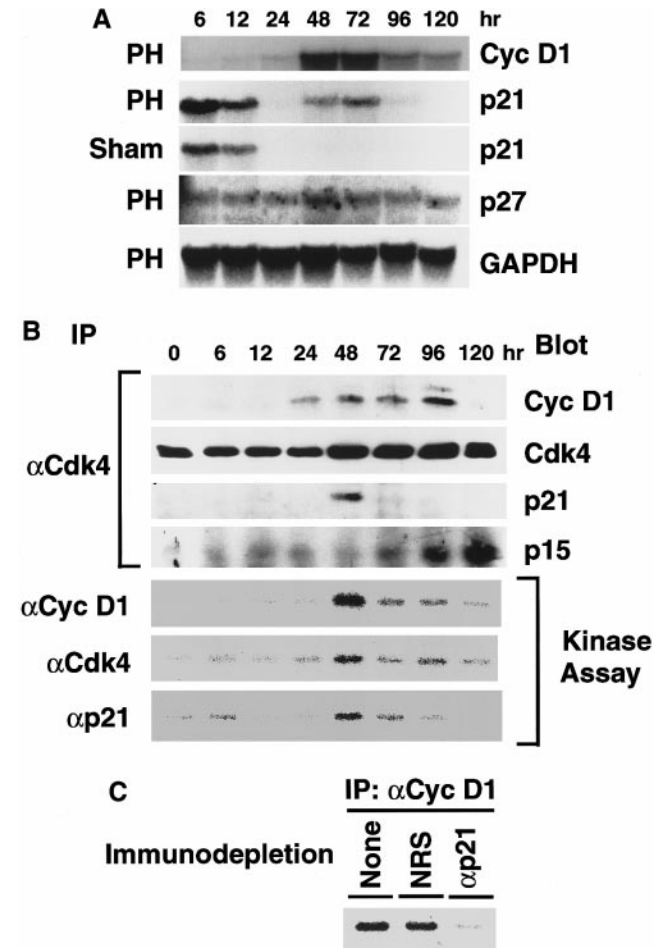

FIG. 3. Cdk4 activation during liver regeneration in the mouse. (A) p21 mRNA was induced in a biphasic manner after partial hepatectomy (PH). Northern blot analysis was performed using total RNA (20 μg) isolated from liver tissues after PH or a sham operation (Sham). Mouse cyclin D1, p21 and p27 cDNAs were used as probes. The intervals following the operation (in hours) are indicated at the top of A, B, and C. (B) Liver extracts (5 mg each of protein) were immunoprecipitated using anti-Cdk4 antibody, and the immunoprecipitates were subjected to Western blotting with antibody against cyclin D1, Cdk4, p21, or p15. Liver extracts were also immunoprecipitated with either anti-cyclin D1, Cdk4, or p21 antibody, and the immunoprecipitates were assayed for Rb kinase activity. (C) The liver extracts at 48 h after PH were precleared twice with either normal rabbit serum (NRS) or anti-p21 antibody and the lysates were then subjected to immunoprecipitation with anti-cyclin D1 antibody, followed by the determination of cyclin D1-associated Rb kinase activity.

ered specific to the liver regeneration process. p27 mRNA levels did not change during the experimental period, and the levels of GAPDH mRNA as an internal control remained almost constant during the liver regeneration process (Fig. 3A).

We next examined the components of the complex formed with Cdk4, and the cyclin D1-, Cdk4-, and p21-associated kinase activities during liver regeneration. Cyclin D1-bound Cdk4 was observed between 24 and 96 h (Fig. 3B, upper panel). It is significant that p21-bound Cdk4 was detected only at 48 h after PH (Fig. 3B, third panel). In contrast, the level of p15-bound Cdk4 remained almost constant until 24 h after PH, slightly decreased at 48 h and then progressively increased (Fig. 3B, fourth panel), suggesting the function of p15 in terminating liver regeneration process. In this regard, it is of interest that TGF-β, which can induce the expression of p15 mRNA, has been implicated as a candidate terminator of the regeneration process (29–31). Interestingly, both cyclin D1- and Cdk4-associated kinase activities peaked at 48 h after PH and immediately decreased thereafter. p21-associated activity was also markedly increased at 48 h after PH, coincident with the second peak of p21, although a smaller peak of activity was also detected at 6 h, which might correspond to the first peak of p21 mRNA (Fig. 3A). Expression of no other known CKIs of the INK4 family was observed in regenerating liver either by Northern or Western analysis (data not shown). Taken together, these results suggest that Cdk4-associated kinase activity is attributable to cyclin D1/Cdk4/p21 ternary complexes during liver regeneration *in vivo*. Immunodepletion experiments revealed that at 48 h after PH, the majority of cyclin D1-associated kinase activity contained p21 (Fig. 3D).

Collaboration between cyclin D1 and p21 in the activation of p15-bound Cdk4 in vitro. Since most Cdk4 binds p15 when cells are in a quiescent state (Figs. 1A and 1C), dissociation of p15 and Cdk4 complex formation with cyclin D are considered to be critical for activation. We therefore examined whether cyclin D1 or p21 could dissociate p15 from Cdk4. To perform this experiment under physiological ratio of p21/p15, we first determined the amounts of p21, and p15 proteins at 4 h after simulation in Bac1.2F5 cells, whose timing is correlated with that of Cdk4 activation (Fig. 1). Using GST-fused p21 and p15 proteins as standards, the amount of p21 and p15 proteins was calculated at 4 and 8 ng/1 $\times$ 10^6 cells, respectively. Based on this calculation, effects of p21 produced in insect cells on both interactions of cyclinD1 and p15 with Cdk4, and Cdk4 kinase activity was then examined. Results showed that neither cyclin D1 nor p21 affected the Cdk4/p15 interaction, and that the addition of cyclin D1 to the Cdk4/p15 complex yielded a cyclin D1/Cdk4/p15 ternary complex (Fig. 4 and data not shown). Thus,

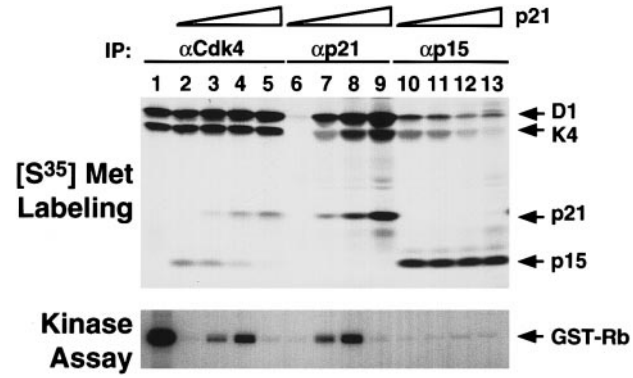

FIG. 4. Collaboration between cyclin D1 and p21 in dissociating p15 and formation of active cyclin D1/Cdk4/p21 complexes. The mixtures of [^{35}S]-labeled insect cell lysates expressing cyclin D1/Cdk4 and p15 (30 μl each) were incubated with increasing amounts of [^{35}S]-labeled insect cell lysates expressing wild-type p21 (0 μl for lanes 2, 6, and 10: 5 μl for lanes 3, 7, and 11: 25 μl for lanes 4, 8, and 12: 125 μl for lanes 5, 9, and 13). The concentration of p21 and p15 in insect cell lysates were calculated at 25 ng/μl, and 20 ng/μl, respectively, using GST-fused p21 and p15 proteins as standards. The mixtures were immunoprecipitated using antibody against Cdk4, p21 or p15. The immunoprecipitates were separated by SDS–PAGE (12.5%), and proteins were detected by autoradiography (upper panel). Aliquots of the immunoprecipitates described were assayed for kinase activity using GST-Rb as a substrate (bottom panel).

it is suggested that neither cyclin D1 nor p21 alone is sufficient for the dissociation of p15.

In contrast, the addition of p21 to the cyclin D1/Cdk4/p15 complex caused an effective and dose-dependent dissociation of p15, resulting in the formation of a cyclin D1/Cdk4/p21 ternary complex (Fig. 4, upper panel). This dose-dependent exchange between p15 and p21 in the ternary complex resulted in an activation of Cdk4 complexes at low concentrations of p21 and inhibition of kinase activity at high concentrations (Fig. 4, bottom panel).

In conclusion, our results clearly indicates the novel function of p21 as a "releaser" of INK4 CKIs from Cdk4 during G1 progression both *in vitro* and *in vivo* systems. The most significant aspect of this model is that p21 forms a kinase-active complex with cyclin D1/Cdk4. Although several hypotheses have been proposed, the exact mechanism by which p21 forms an active complex with cyclin/Cdk remains to be elucidated. There are a number of situations in which p21 induction has been associated with cell cycle progression or cellular proliferation (21, 22, 32–35). The most striking example reported is that as much as a 10-fold induction of p21 was observed in a myeloid cell line stimulated to proliferate by Steel factor and granulocyte-macrophage colony-stimulating factor (36). Thus, the cooperation of p21 with cyclin D may represent a general mechanism for Cdk4 activation during G1 progression.

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