

# Rapamycin Disrupts Cyclin/Cyclin-Dependent Kinase/p21/Proliferating Cell Nuclear Antigen Complexes and Cyclin D1 Reverses Rapamycin Action by Stabilizing These Complexes

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## Abstract

Rapamycin and its derivatives are promising anticancer agents, but the exact mechanisms by which these drugs induce cell cycle arrest and inhibit tumor growth are unknown. A biochemical analysis of human mammary tumor cell lines indicated that rapamycin-induced antiproliferative effects correlated with down-regulation of cellular p21 levels and the levels of p21 in cyclin-dependent kinase (Cdk) 2 and 4 complexes. Cyclin D1 overexpression reversed rapamycin action and this reversal correlated with increased levels of cellular p21, higher levels of p21 associated with Cdk2, and stabilization of cyclin D1/Cdk2/p21/proliferating cell nuclear antigen (PCNA) complexes. Experiments using a novel cyclin D1-Cdk2 fusion protein or a kinase-dead mutant of the fusion protein indicated that reversal of rapamycin action required not only the formation of complexes with p21 and PCNA but also complex-associated kinase activity. Similar results were observed *in vivo*. The rapamycin derivative RAD001 (everolimus) inhibited the growth of mouse mammary tumors, which correlated with the disruption of cyclin D1/Cdk2 complexes. The potential implications of these results with respect to the use of rapamycin derivatives in breast cancer therapy are discussed. (Cancer Res 2006; 66(2): 1070-80)

## Introduction

Rapamycin derivatives, such as RAD001 (everolimus) and CCI-779, are currently in clinical trials as anticancer agents. Rapamycin inhibits the mammalian target of rapamycin (mTOR) protein kinase resulting in decreased phosphorylation and altered activity of p70<sup>S6k</sup> and 4EBP1, the most extensively studied downstream effectors of mTOR. However, the exact mechanisms by which rapamycin mediates cancer cell cycle arrest and the factors that control tumor sensitivity to rapamycin action are poorly understood.

It is unclear why rapamycin inhibits the phosphorylation of p70<sup>S6k</sup> and 4EBP1 nearly universally, whereas sensitivity to rapamycin antiproliferative effects varies dramatically among different tumor cell lines. Rapamycin induces a G<sub>1</sub> cell cycle arrest and this correlates with down-regulation of cyclin D1 levels in

some cell types (1, 2) and cyclin D1 overexpression reverses rapamycin-induced cell cycle arrest (3). The latter observation may be particularly significant with respect to the use of rapamycin derivatives in breast cancer therapy because 40% to 50% of human mammary carcinomas exhibit cyclin D1 overexpression (4, 5).

In this study, we examined the mechanisms by which cyclin D1 overexpression reverses rapamycin-induced cell cycle arrest and the effect of rapamycin treatment on the subunit structure of cyclin-dependent kinase (Cdk) complexes containing Cdk2 and Cdk4.

## Materials and Methods

**Cell culture, construction of stable cell lines, [<sup>3</sup>H]thymidine incorporation assays, and flow cytometry.** BT549, MDA-MB-361, MCF10A, NMuMG, T47D, MDA-MB-435S, MDA-MB-468, MDA-MB-231, MDA-MB-436, HBL100, and MCF7 cells were obtained from the American Type Culture Collection (Rockville, MD). Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD) provided p21-deficient HCT116 cells. Polyclonal stable cell lines overexpressing cyclin D1 were prepared as described previously using a recombinant cyclin D1 retrovirus followed by selection with 1.5 µg/mL puromycin (6). The retrovirus encodes mouse cyclin D1 with a T286A mutation (7). In control experiments, wild-type mouse cyclin D1 and the T286A mutant exhibited identical effects on rapamycin responses when corrected for differences in the levels of protein expression. Flow cytometry analyses of propidium iodide-stained cells and data modeling were done by the University of Florida Shands Cancer Center Flow Cytometry Core Facility.

**Immunoprecipitation and immunoblot analysis of mouse mammary tumor extracts.** A representative sample of ~50 mg tumor tissue was sonicated in extraction buffer [20 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1% β-mercaptoethanol, 5% glycerol, 1 mmol/L microcystin, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 40 mmol/L Na<sub>2</sub>H<sub>2</sub>P<sub>2</sub>O<sub>7</sub>] containing 1% Triton X-100 and the extract was clarified by centrifugation. Protein concentrations were determined using the Bradford assay, and the extracts were diluted in extraction buffer containing 0.1% Triton X-100 to 2 mg/mL. For each immunoprecipitation, protein (2 mg) was precleared twice with 50 µL packed protein G-Sepharose resin (Zymed Laboratories, Inc., San Francisco, CA) and the supernatant was subjected to immunoprecipitation with 4 µg antibody or the appropriate control preimmune IgG. In immunoblot analyses of crude tumor protein extracts, protein (30 µg) was loaded per lane. Preparation of cell extracts, immunoblot, and immunoprecipitation were done as described previously (8, 9).

**Construction of recombinant adenoviruses.** Adenoviruses encoding green fluorescent protein (Ad.GFP), cyclin D1-Cdk2 fusion protein (Ad.D1K2), His<sub>6</sub>-tagged p27 (Ad.p27), and Cdk2-His<sub>6</sub> (Ad.Cdk2) were described previously (9). Adenoviruses encoding a kinase-dead mutant of the cyclin D1-Cdk2 fusion protein [Ad. D1K2(KD); ref. 9] and His<sub>6</sub>-tagged p21 were prepared using established methods (10). Plaque-forming units of adenovirus were estimated by the 50% tissue culture infectious dose

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statistically using Karber's formula, with modifications. Wells of 293 cells were scored positive if any GFP-positive cells were observed. Viral dilutions were done in serum-free medium containing 10  $\mu\text{g}/\text{mL}$  polybrene to increase the efficiency of adenoviral infection (11). GFP fluorescence was recorded using a CKX41 microscope equipped with a DP12 digital camera and a U-RELT50 UV source (Olympus, Melville, NY).

**Mouse mammary tumor studies.** MMTV-*c-neu* mice (12) were provided by Dr. Harold L. Moses (Vanderbilt-Ingram Cancer Center, Nashville, TN). In the tumor studies, tumor-bearing female mice were randomly assigned to the vehicle or RAD001 treatment groups when palpable tumors were detected. The animals were treated for 5 consecutive days followed by 2 days with no treatment, and successive cycles were repeated throughout the treatment period. The 2 days in each cycle in which the mice were not treated are not included in the number of treatment days. RAD001 emulsion was diluted in sterile water and 100  $\mu\text{L}$  were administered by oral gavage to achieve a dose of 2.5 mg/kg. The vehicle control was administered in an identical manner. Tumor dimensions were measured using calipers and tumor volumes were calculated using the formula:  $(W^2 \times L) / 2$ , where width ( $W$ )  $\leq$  length ( $L$ ).

In biochemical analyses where several milligrams of tumor tissue were required, tumors were allowed to grow until reaching 0.5 cm before treatment with vehicle or 2.5 mg/kg RAD001 was initiated. Mice were treated for 5 consecutive days and tumor tissue was harvested on the fifth day 4 hours after treatment. Tumor extracts were prepared and analyzed as described above.

**Statistics.**  $P$ s were calculated using the GraphPad.com online calculator to make pairwise comparisons between the means of the indicated groups in an unpaired Student's  $t$  test.  $P$ s  $<$  0.05 were considered statistically significant. Tumor growth curves were fitted to a line by least-squares analysis using CA-Cricket Graph III (Computer Associates International, Islandia, NY).

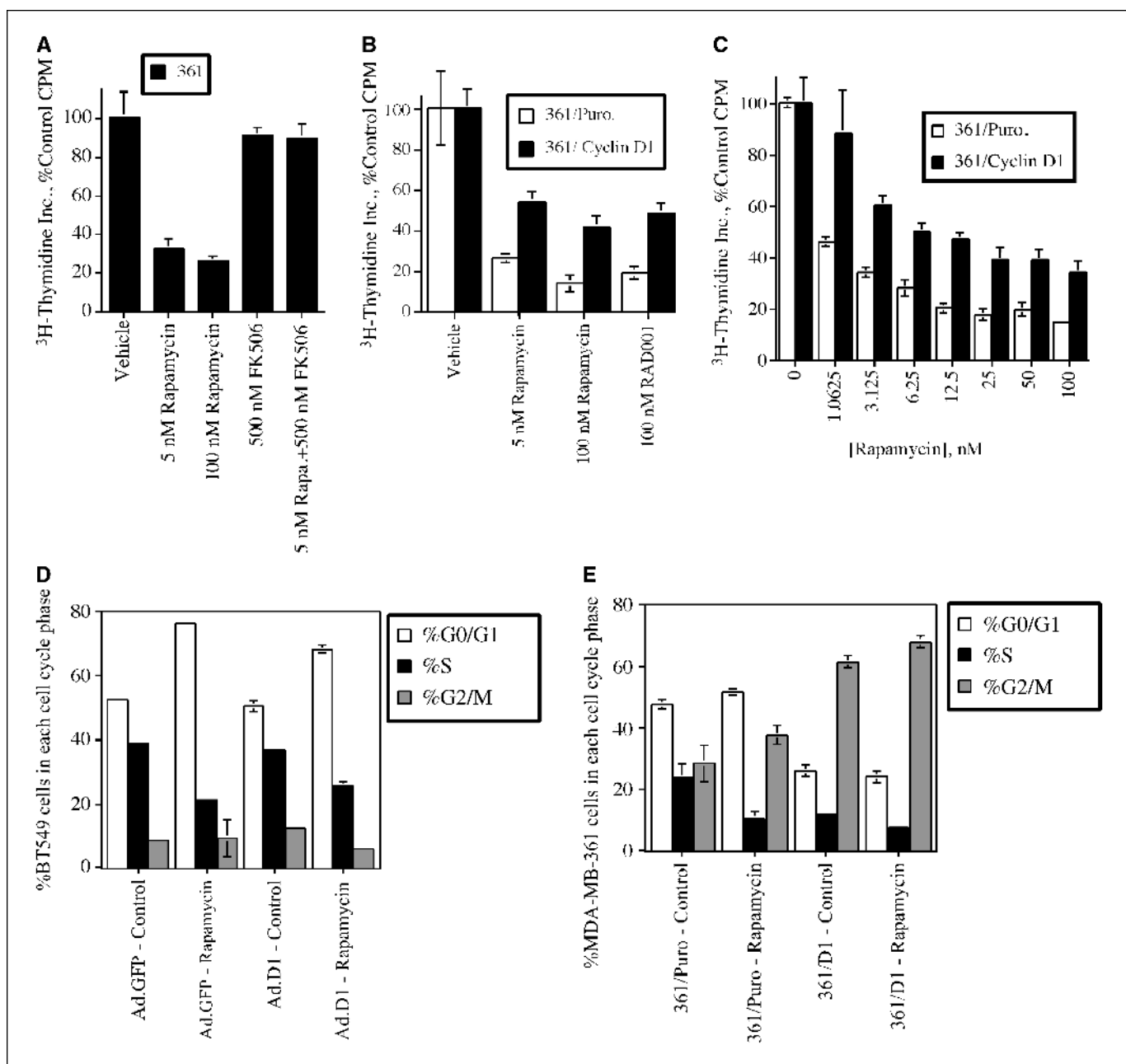
## Results

**Cyclin D1 suppression of rapamycin-induced cell cycle arrest.** The rapamycin sensitivity of several human breast cancer cell lines was characterized to find a suitable model system in which to study the mechanisms of rapamycin action. The MDA-MB-361 and BT549 metastatic human mammary carcinoma cell lines were chosen for further study because of their high sensitivity to rapamycin-induced cell cycle arrest. Rapamycin (5 nmol/L) inhibited DNA synthesis in MDA-MB-361 cells by  $\sim$  70% (Fig. 1A), and the inhibition was reversed by FK506 (500 nmol/L), which prevents binding of rapamycin to its intracellular receptor FKBP12. The rapamycin derivative RAD001 and rapamycin inhibited the proliferation of MDA-MB-361 cells to similar extents at a concentration of 100 nmol/L (Fig. 1B).

Cyclin D1 is down-regulated by rapamycin in some cell types (1, 2), and cyclin D1 overexpression reverses rapamycin-induced cell cycle arrest (3), so we constructed cell lines to examine the effects of stable overexpression of cyclin D1 on biological and biochemical responses to rapamycin. Polyclonal stable cell lines were constructed by transduction of MDA-MB-361 cells with a control retroviral vector (361/Puro) or a retroviral vector encoding cyclin D1 (361/cyclin D1) followed by selection with puromycin. MDA-MB-361 cells constitutively overexpressing cyclin D1 were significantly less sensitive to rapamycin and RAD001 inhibition of cell proliferation (Fig. 1B) compared with the control cells, and this difference in sensitivity was observed over a broad range of rapamycin concentrations (Fig. 1C). Flow cytometry analyses of propidium iodide-stained cells were done to determine how rapamycin and cyclin D1 overexpression alter cell cycle distribution. Rapamycin induced an increase in the fraction of cells in the  $G_1$  phase of the cell cycle and a corresponding decrease in the

proportion of cells in S phase in BT549 cells (Fig. 1D). Adenovirus-mediated overexpression of cyclin D1 did not significantly affect the cell cycle distribution of BT549 cells but weakened the  $G_1$  arrest induced by rapamycin. Similar analyses of control MDA-MB-361 cells (361/Puro) and cyclin D1-overexpressing MDA-MB-361 cells (361/cyclin D1) indicated that cyclin D1 overexpression induced a dramatic alteration in cell cycle distribution with a decrease in the fraction of cells in  $G_1$  and a corresponding increase in the fraction of cells in  $G_2$ . In MDA-MB-361 cells, the rapamycin-induced decrease in the S-phase fraction was associated with an increase in the proportion of cells in both  $G_1$  and  $G_2$  phases. Cyclin D1 overexpression blunted the decrease in the fraction of cells in S phase in response to rapamycin treatment. This effect was primarily due to the inability of rapamycin to increase the fraction of cells in  $G_1$  in the context of cyclin D1 overexpression. In contrast, the rapamycin-induced increase in the fraction of cells in  $G_2$  was unaltered by cyclin D1 overexpression. Cyclin D1 was shown previously to control the rate at which cells traverse the  $G_1$  phase of the cell cycle (13). Together, the results in Fig. 1 are most consistent with a model in which rapamycin induces a  $G_1$  cell cycle arrest and high cyclin D1 levels override rapamycin-induced cell cycle arrest and alter the cell cycle distribution in cells in which cyclin D1 levels are limiting for cell proliferation.

**Cyclin D1 increases p21 levels and rapamycin decreases p21 levels.** Previous studies have indicated that in various cell lines rapamycin decreases cellular levels of cyclin D1, proliferating cell nuclear antigen (PCNA), p21, and c-Myc (1-3, 14-18). Other studies have identified cellular complexes containing cyclin D1, Cdk2, PCNA, and p21 (9, 19, 20). Together, these observations suggest that cyclin D1/Cdk2/p21/PCNA complexes might be important targets of rapamycin action. To test this hypothesis, control or cyclin D1-overexpressing MDA-MB-361 cells were treated with low (1 or 5 nmol/L) or high (100 nmol/L) concentrations of rapamycin and cell extracts were prepared for immunoblot analysis. The results of two replicate experiments are shown and indicate that, as expected, rapamycin blocked the phosphorylation of p70<sup>S6k</sup> but did not affect the phosphorylation of mitogen-activated protein kinase (Fig. 2A). Cyclin D1 overexpression did not override the ability of rapamycin to block p70<sup>S6k</sup> phosphorylation. This result indicates that cyclin D1 effects are mediated downstream of the mTOR/p70<sup>S6k</sup> signaling cascade likely at the level of the cell cycle. Neither total PCNA nor c-Myc levels were strongly affected by rapamycin in either the control or cyclin D1-overexpressing cell line. Cyclin D1 was down-regulated by rapamycin in some experiments (e.g., Fig. 2A, left) but not in other experiments (e.g., Fig. 2A, right). Rapamycin had no effect on retrovirus-mediated cyclin D1 overexpression. This is likely because the overexpressed cyclin D1 contains the T286A mutation that prevents its phosphorylation by glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) and subsequent proteolytic degradation (21) and is consistent with previous observations (22). In contrast, rapamycin induced a dose-dependent decrease in p21 levels in both the presence and the absence of cyclin D1 overexpression. Strikingly, cyclin D1 overexpression seemed to stabilize p21 levels as shown by the fact that p21 levels in the 361/cyclin D1 cells were much higher than in the control cells. Rapamycin induced p21 down-regulation in the 361/cyclin D1 cells, but p21 levels in 361/cyclin D1 cells treated with 100 nmol/L rapamycin were similar to the p21 levels present in the 361/Puro cells in the absence of rapamycin. Thus, in this sense, cyclin D1 overexpression effectively reversed rapamycin-induced p21 down-regulation because of the high basal levels of p21 in the context of cyclin D1 overexpression.



**Figure 1.** Cyclin D1 overexpression suppresses rapamycin- and RAD001-induced inhibition of cell proliferation. *A*, MDA-MB-361 cells (361) were treated for 24 hours with the indicated concentrations of rapamycin and FK506. The cells were pulsed for 2 hours with [<sup>3</sup>H]thymidine, and [<sup>3</sup>H]thymidine incorporation was quantitated using a scintillation counter. *B* and *C*, MDA-MB-361 cells were transduced with an empty retrovirus as a control (361/Puro.) or a retrovirus encoding cyclin D1 (361/Cyclin D1). The cells were treated for 24 hours with the indicated concentrations of rapamycin or RAD001 and [<sup>3</sup>H]thymidine incorporation was quantitated by scintillation counting. *Columns*, mean [<sup>3</sup>H]thymidine incorporation of assays done in triplicate and normalized to the vehicle control; *bars*, SD. *D*, BT549 cells were infected with adenoviruses encoding GFP (*Ad.GFP*) or cyclin D1 (*Ad.D1*) at MOIs of 1,000 and incubated for 24 hours. The cells were treated for an additional 24 hours with or without 100 nmol/L rapamycin. Cells were stained with propidium iodide and analyzed by flow cytometry. *Columns*, average of triplicate determinations; *bars*, SD. *E*, control MDA-MB-361 cells (361/Puro) or MDA-MB-361 cells overexpressing cyclin D1 (361/D1) were treated for 24 hours with or without 100 nmol/L rapamycin. Results were analyzed and presented as in (*D*).

These results suggested that there may be a relationship between cyclin D1 levels, p21 levels, and rapamycin sensitivity.

Analysis of multiple mammary epithelial and mammary carcinoma cell lines indicated that in general cell lines with low levels of either p21 or cyclin D1 (e.g., MDA-MB-361, MCF7, BT549, and NMuMG) exhibit high sensitivity to rapamycin, whereas cells expressing high levels of both cyclin D1 and p21 (e.g., MDA-MB-231, MDA-MB-436, and MCF7) exhibit low sensitivity to rapamycin

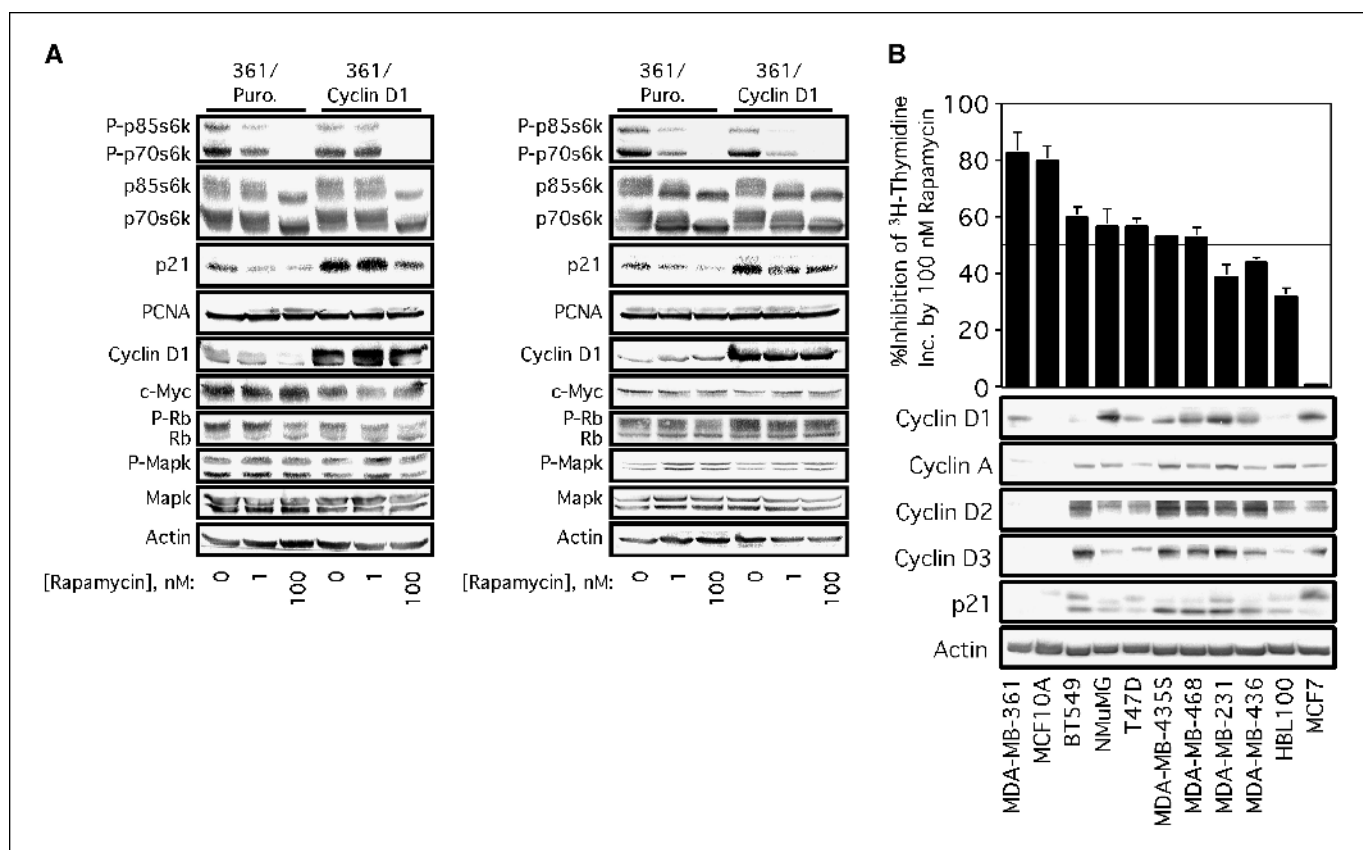
(Fig. 2*B*). The only obvious exception is the HBL100 cell line. HBL100 cells express high levels of cyclin D2, cyclin D3, and cyclin A, which may confer rapamycin resistance in the absence of cyclin D1 (23, 24). Alternatively, additional mechanisms may be responsible for the rapamycin resistance of HBL100 cells. Most striking is the finding that the two cell lines with the highest rapamycin sensitivity, MDA-MB-361 and MCF10A, express low levels of p21 as well as low levels of multiple cyclins. In contrast,

cell lines expressing high levels of both cyclin D1 and p21 (e.g., MCF7, MDA-MB-436, MDA-MB-231, and MDA-MB-468) exhibit rapamycin resistance. There were no instances in which a cell line expressed high levels of both cyclin D1 and p21 and exhibited high sensitivity to rapamycin. The results in Fig. 2 are consistent with a model in which cyclin D1 and p21 function together to mediate cellular resistance to rapamycin-induced arrest.

Several observations suggest that rapamycin-induced p21 down-regulation is as relevant to rapamycin action as cyclin D1 down-regulation. In the context of cyclin D1 overexpression, rapamycin did not affect cyclin D1 levels, but rapamycin still decreased p21 levels, and this correlated with the somewhat dampened inhibition of proliferation that occurred even in the presence of cyclin D1 overexpression. This result suggests that a major role for cyclin D1 is to stabilize p21 and perhaps p21-containing complexes. Consistent with this contention, E2F2 overexpression in hepatocytes reversed rapamycin-induced cell cycle arrest, and this correlated with restoration of p21 and PCNA expression but not cyclin D1 expression (3). Dong et al. showed recently that rapamycin induces cyclin D1 down-regulation through the activation of GSK3 $\beta$  (22). Inhibition of GSK3 $\beta$  with the specific inhibitor SB216763 blocked the ability of rapamycin to induce cyclin D1 down-regulation but did not block rapamycin-induced G<sub>1</sub> cell cycle arrest. Taken together, these observations suggest that

p21, in addition to cyclin D1, may be an important cell cycle target of rapamycin action.

**Rapamycin disrupts Cdk2/PCNA complexes.** We showed previously that p21 facilitates the formation of complexes between Cdk2 and PCNA in HCT116 colon carcinoma cells (9). p21 was not absolutely required for the formation of complexes containing cyclin D1 and Cdk2. However, lower levels of cyclin D1/Cdk2 complexes were present in p21-deficient cells, suggesting that p21 stabilizes cyclin D1/Cdk2 complexes. Based on these observations, we hypothesized that rapamycin would disrupt Cdk2 interaction with PCNA by inducing p21 down-regulation and might also significantly affect the interaction between Cdk2 and cyclin D1. To test this hypothesis, Cdk2 or Cdk4 were immunoprecipitated from control cells or cyclin D1-overexpressing cells treated with or without rapamycin (Fig. 3A). The results indicate that rapamycin not only down-regulated levels of total cellular p21 (Fig. 2A) but also down-regulated the levels of p21 associated with Cdk2 and Cdk4. Consistent with the results in Fig. 2, cyclin D1 overexpression reversed the rapamycin-induced decrease in Cdk2- and Cdk4-associated p21 levels. PCNA was present in Cdk2 complexes but was not observed in Cdk4 complexes in MDA-MB-361 cells. Together, the results indicate that rapamycin decreased the levels of p21 present in Cdk2 and Cdk4 complexes, and this was paralleled by a loss of PCNA in Cdk2 complexes. This is consistent with our

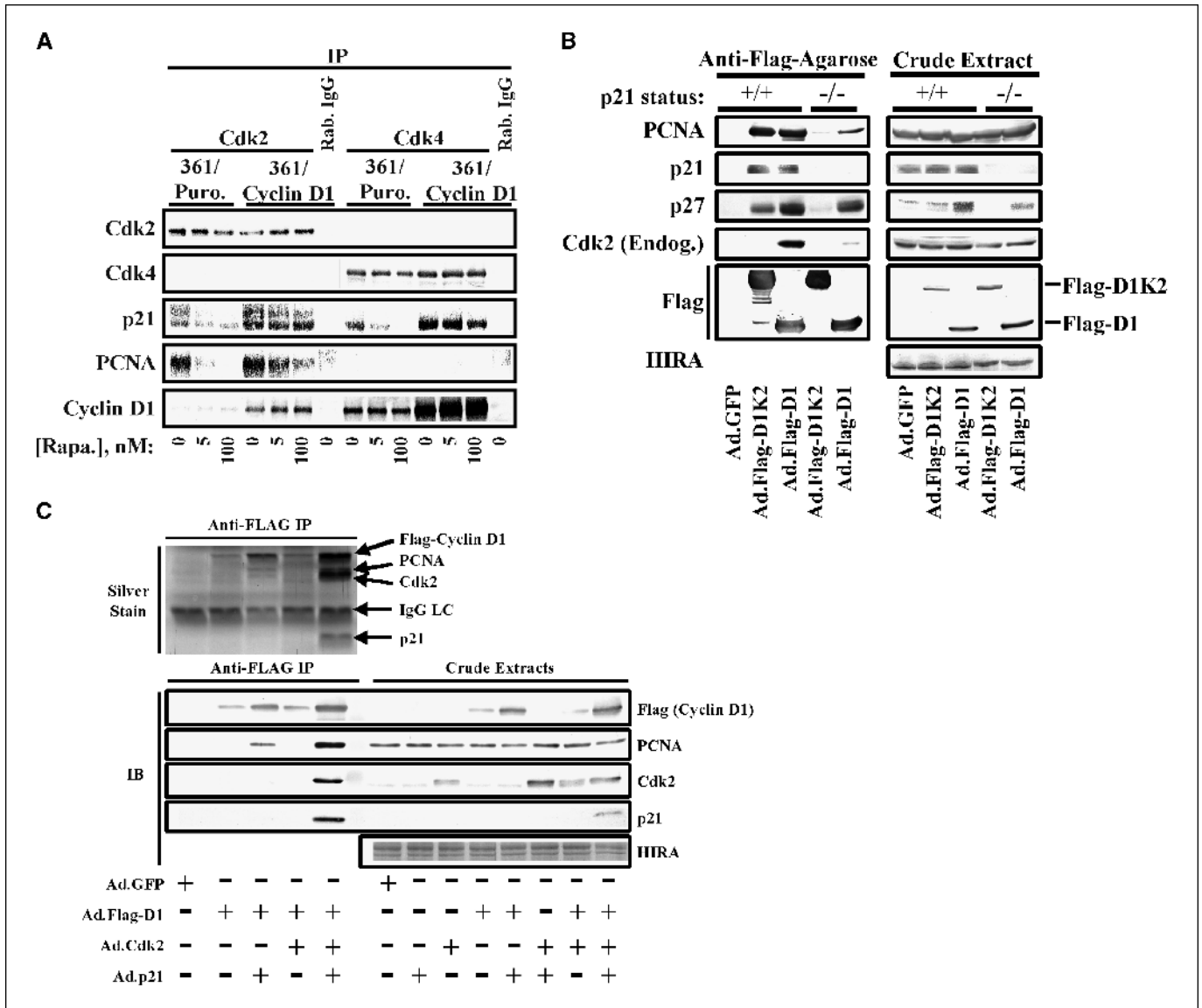


**Figure 2.** Cellular p21 levels are down-regulated by rapamycin and up-regulated by cyclin D1. *A*, control or cyclin D1-overexpressing MDA-MB-361 cells were treated for 24 hours with either 0, 1, or 100 nmol/L rapamycin (*left*) or 0, 5, or 100 nmol/L rapamycin (*right*). Cell extracts were prepared and analyzed by immunoblot as described in Materials and Methods using the indicated antibodies. *p85<sup>s6k</sup>* and *p70<sup>s6k</sup>*, alternate translation products of the same mRNA; *P-p85<sup>s6k</sup>* and *P-p70<sup>s6k</sup>*, *p85<sup>s6k</sup>* and *p70<sup>s6k</sup>* proteins phosphorylated on Thr<sup>389</sup>; *P-Rb*, hyperphosphorylated forms of Rb that exhibit retarded electrophoretic mobility. *B*, *top*, the indicated cell lines were treated for 24 hours with or without 100 nmol/L rapamycin and subjected to [<sup>3</sup>H]thymidine incorporation assays. *Columns*, percent inhibition of [<sup>3</sup>H]thymidine incorporation assays by rapamycin for each cell line done in triplicate; *bars*, SD. *Bottom*, cell lysates from each of the indicated cell lines were prepared and analyzed by immunoblot using antibodies specific for cyclin D1, cyclin A, cyclin D2, cyclin D3, p21, or actin as a loading control.

previous study showing that p21 facilitates the formation of complexes containing both Cdk2 and PCNA (9). Rapamycin maintained the dissociation of complexes between Cdk2 and PCNA for up to 72 hours, the longest time point examined (data not shown).

**p21, but not p27, facilitates the formation of cyclin D1/Cdk2 complexes.** Cyclin D1 and Cdk2 both bind to p27, making it possible that p27 might substitute for p21 in facilitating the formation of cyclin D1/Cdk2 complexes. In addition, p21-null mice are viable so the formation of complexes containing PCNA and Cdk2 is either not required for cell proliferation or these complexes form at a much lower level that allows cell proliferation albeit at a suboptimal rate. These issues were addressed in analyses of cyclin

D1-containing complexes carried out in parental or p21-deficient HCT116 cells. Parental cells (+/+) or p21-null cells (-/-) were infected with adenoviruses encoding GFP, Flag-tagged cyclin D1-Cdk2 fusion protein, or Flag-tagged cyclin D1 (Fig. 3B). This fusion protein consists of a FLAG epitope tag followed by the cyclin D1 domain, a flexible linker, the Cdk2 domain, and a His<sub>6</sub> affinity tag at the COOH terminus. We have shown previously that the cyclin D1-Cdk2 fusion protein is catalytically active and interacts with p21 and PCNA, the same proteins present in native cyclin D1/Cdk2 complexes (9). Immunoblot analysis of material purified by anti-Flag-agarose affinity chromatography indicated that in the presence of normal p21 levels the cyclin D1-Cdk2 fusion protein



**Figure 3.** Rapamycin (*Rapa.*) destabilizes cyclin D1/Cdk2/p21/PCNA complexes. *A*, extracts from cells treated as in Fig. 2 were subjected to immunoprecipitation using antibodies specific for Cdk2, Cdk4, or preimmune rabbit IgG (*Rab. IgG*) as a control. The resulting immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. *B*, parental HCT116 cells (p21<sup>+/+</sup>) or p21-deficient HCT116 cells (p21<sup>-/-</sup>) were infected with adenoviruses encoding GFP, Flag-tagged cyclin D1-Cdk2 fusion protein (*Ad.Flag-D1K2*), or Flag-tagged cyclin D1 (*Ad.Flag-D1*) at a MOI of 1,000. Forty-eight hours after infection, cell extracts were prepared and analyzed by immunoblot or subjected to affinity chromatography using anti-Flag-agarose resin followed by immunoblot analysis with the indicated antibodies. *Cdk2* (*Endog.*), levels of endogenous Cdk2 expression (*right*) or level of endogenous Cdk2 association with Flag-tagged cyclin D1 (*left*). Immunoblot with the anti-Flag antibody was used to detect the Flag-tagged cyclin D1-Cdk2 fusion protein (*Flag-D1K2*) and Flag-tagged cyclin D1 (*Flag-D1*) as indicated. *C*, BT549 cells were treated with adenoviruses encoding GFP, Flag-tagged cyclin D1, Cdk2 (*Ad.Cdk2*), or p21 (*Ad.p21*) as indicated at a MOI of 100 for each virus and incubated for 48 hours. Cell lysates were prepared and a portion was analyzed directly by immunoblot (*Crude Extracts*). The remainder was subjected to affinity purification using anti-Flag-agarose. Results were analyzed by silver stain (*top*) or by immunoblot with antibodies specific for the Flag tag, PCNA, Cdk2, p21, or HIRA as a loading control.

interacted with PCNA, p21, and p27. Under the same conditions, cyclin D1 interacted with similar amounts of PCNA and p21 and a slightly greater amount of p27. This apparently resulted from increased expression of p27 caused by cyclin D1 overexpression (Fig. 3B, right). In the absence of p21, the cyclin D1-Cdk2 fusion protein bound a very small, but detectable, amount of PCNA and bound lower levels of p27 apparently due to decreased levels of p27 expression in p21-null cells. Cyclin D1 bound PCNA in the absence of p21 but at levels much lower than those observed in the presence of normal p21 expression. Strikingly, cyclin D1 bound much less Cdk2 in the absence of p21 expression. Cyclin D1 bound relatively high levels of p27 in p21-null cells. The amount of Cdk4 bound by cyclin D1 in the presence or absence of p21 was the same (data not shown). Together, these results indicate that cyclin D1/Cdk2/PCNA-containing complexes are particularly sensitive to cellular p21 levels and that p27 is unable to substitute for p21 as an assembly factor for these complexes. By extension, because rapamycin decreases p21 levels (Fig. 2A), these results explain how rapamycin decreases PCNA binding to Cdk2. In MDA-MB-361 cells, rapamycin only decreased PCNA association with Cdk2 but did not alter the association between cyclin D1 and Cdk2. In p21-null HCT116 cells, cyclin D1 did not interact strongly with either Cdk2 or PCNA. The reason for these differences is unclear, but several possibilities exist. There may be cell type-specific differences in the formation of these complexes in HCT116 versus MDA-MB-361 cells. The manner in which the complexes were isolated may account for the differing results. Complexes in MDA-MB-361 cells were isolated by immunoprecipitation with Cdk2 antibodies, whereas complexes in BT549 cells were isolated using adenovirally expressed Flag-cyclin D1 followed by purification using anti-Flag-agarose resin. Optimal complex formation between cyclin D1 and PCNA may require higher p21 levels than the formation of complexes between cyclin D1 and Cdk2. If this were the case, it might explain why rapamycin, which partially decreases p21 levels, only disrupts complexes between Cdk2 and PCNA without affecting complexes between Cdk2 and cyclin D1, whereas a complete deficiency of p21 strongly suppresses the formation of complexes between Cdk2 and both cyclin D1 and PCNA. Such a scenario would also suggest that the growth inhibitory effects of rapamycin and its effects on cyclin D1/Cdk2/p21/PCNA-containing complexes may be predicted by cellular levels of both p21 and cyclin D1 and the degree to which rapamycin suppresses the levels of these proteins.

**Mutual stabilization of the components of cyclin D1/Cdk2/p21/PCNA complexes.** Rapamycin has been reported to decrease cellular levels of cyclin D1, p21, and PCNA in various studies (1–3, 14–18, 25). Figures 2A and 3A indicated that cyclin D1 overexpression increased p21 levels and made cyclin D1/Cdk2/p21/PCNA complexes resistant to rapamycin-induced disruption. Based on these observations, we hypothesized that complex formation between cyclin D1, Cdk2, p21, and PCNA stabilizes each of these components and that the total cellular levels of the individual protein subunits and the levels of the complexes that they participate in are interdependent. This hypothesis was tested using recombinant adenoviruses to overexpress p21, Flag-tagged cyclin D1, or Cdk2 in BT549 cells separately or in various combinations. The formation of complexes was monitored by silver stain and immunoblot analyses of anti-Flag-agarose immunoprecipitates, and total protein levels were determined by immunoblot analyses of crude extracts (Fig. 3C). Flag-cyclin D1 expressed alone or coexpressed with Cdk2 did not associate with

a detectable amount of endogenous p21 or PCNA. When p21 was coexpressed with Flag-cyclin D1, the association of endogenous PCNA was evident, but the levels of associated p21 and Cdk2 were too low to detect. Coexpression of Flag-cyclin D1, Cdk2, and p21 resulted in complexes containing high levels of all four components, cyclin D1, PCNA, p21, and Cdk2. This result was not simply a matter of expressing the complex components because the associated PCNA was endogenous, and the level of p21 associated was much higher when coexpressed with Flag-cyclin D1 and Cdk2 rather than with just Flag-cyclin D1 alone. Analyses of the crude extracts indicated that p21 overexpression increased the level of coexpressed Flag-cyclin D1. Interestingly, p21 expression in the crude extracts was only detectable when it was coexpressed with both cyclin D1 and Cdk2. For each virus, the same dose was used in the different experimental samples, and the adenoviral expression of all of these proteins was driven by the cytomegalovirus promoter. Thus, the simplest explanation for these results is that cyclin D1 and p21 are more stable when present in cyclin D1/Cdk2/p21/PCNA complexes than in their monomeric forms. This would suggest that overexpression of either protein mutually stabilizes the other through recruitment into multiprotein complexes, with the caveat that sufficient levels of an endogenous Cdk and PCNA must be present. These findings also suggest that relatively small increases in cellular levels of cyclin D1, p21, and PCNA may result in dramatic increases in the levels of cyclin D1/Cdk2/p21/PCNA complexes. Conversely, rapamycin induces decreases in the levels of cyclin D1, p21, and PCNA in a cell type-specific manner; therefore, rapamycin may potentially induce disassembly of cyclin D1/Cdk2/p21/PCNA complexes by causing relatively modest decreases in the cellular levels of the individual components.

**Suppression of rapamycin-induced cell cycle arrest by a cyclin D1-Cdk2 fusion protein requires kinase activity.** The results in Figs. 1, 2, and 3 show that rapamycin-induced cell cycle arrest correlates with p21 down-regulation and the loss of p21 and PCNA from Cdk2 complexes. Cyclin D1 reversed these effects of rapamycin by increasing p21 levels and effectively stabilizing cyclin D1/Cdk2/p21/PCNA complexes. It is possible that the formation of cyclin D1/Cdk2/p21/PCNA complexes alone is sufficient to override rapamycin cytostatic effects by serving as a molecular scaffold. However, in addition to complex formation per se, the presence of kinase activity in these complexes may be required to block rapamycin action. We employed catalytically active or kinase-dead cyclin D1-Cdk2 fusion proteins described previously (9) expressed from recombinant adenoviral vectors to address this issue. The previous study employed inactive mutants of the cyclin D1-Cdk2 fusion protein to show that the cyclin D1 domain activates the Rb and histone H1 kinase activity of the Cdk2 domain through an intramolecular mechanism. The cyclin D1-Cdk2 fusion protein forms complexes containing p21 and PCNA similar to those observed with separately encoded cyclin D1 and Cdk2 (9). The catalytically active cyclin D1-Cdk2 fusion protein, but not a catalytically inactive mutant, mediates the phosphorylation of endogenous Rb and directly phosphorylates Rb in *in vitro* kinase assays. This suggested that we could employ catalytically active or kinase-dead mutants of the cyclin D1-Cdk2 fusion protein to determine whether expression of the cyclin D1-Cdk2 fusion protein mimics the action of cyclin D1 overexpression in reversing rapamycin cytostatic action and whether reversal of rapamycin action requires kinase activity. Rapamycin (100 nmol/L) inhibited cell proliferation by >50% in uninfected BT549 cells

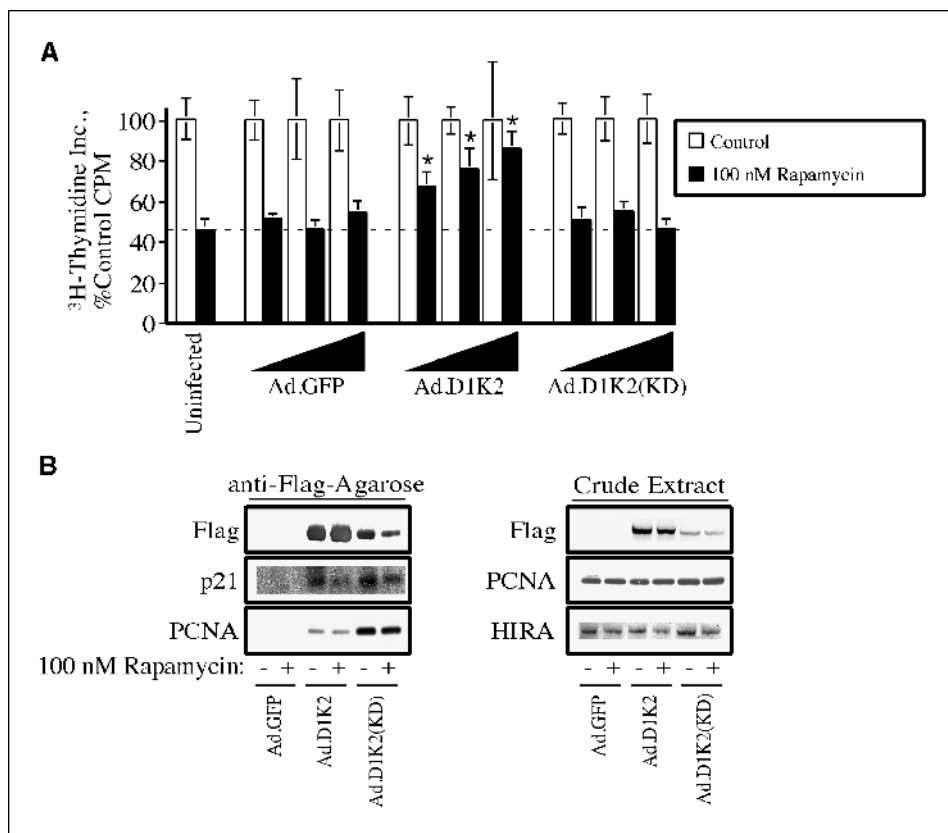
(Figs. 2B and 4A). Increasing concentrations of a GFP adenovirus (Ad.GFP) did not have a significant effect on rapamycin responsiveness. Infection with an adenovirus encoding the catalytically active cyclin D1-Cdk2 fusion protein (Ad.D1K2) dose-dependently reversed rapamycin-induced cell cycle arrest. In contrast, infection with an adenovirus encoding a kinase-dead mutant of the cyclin D1-Cdk2 fusion protein was unable to reverse rapamycin growth inhibition. The observed differences between rapamycin inhibition of proliferation between cells infected with Ad.D1K2 versus uninfected cells or cells infected with Ad.GFP or Ad.D1K2(KD) were statistically significant ( $P < 0.05$ ).

We considered two possible explanations for these observations. The first possibility is that Ad.D1K2(KD) did not reverse rapamycin action because kinase activity is required for this effect. However, a second possibility is that Ad.D1K2(KD) did not reverse rapamycin action because D1K2(KD) does not form complexes with p21 and PCNA as well as the catalytically active D1K2. The experiment in Fig. 4B was done to distinguish between these two possibilities.

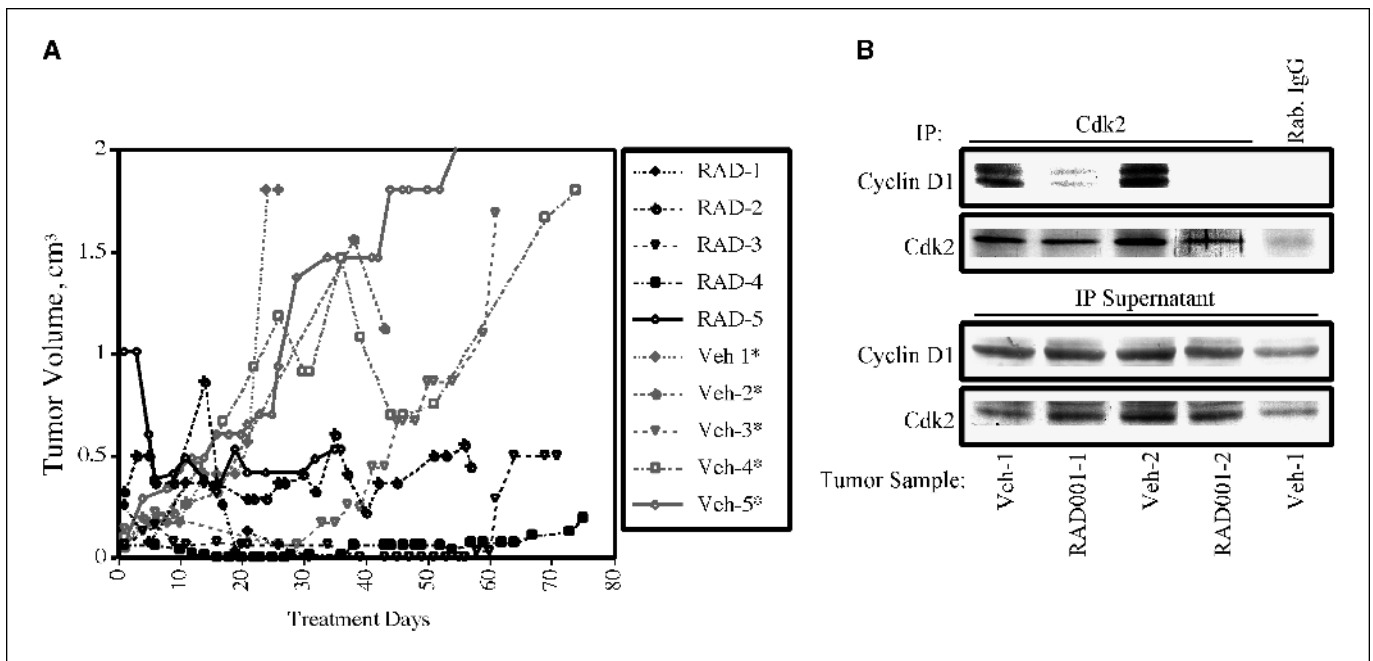
BT549 cells were infected with adenoviruses encoding GFP, D1K2, or D1K2(KD), and cell extracts were prepared and analyzed directly by immunoblot (Fig. 4B, right) or subjected to affinity chromatography using anti-Flag-agarose to isolate the Flag-tagged fusion proteins (Fig. 4B, left). The results indicate that both catalytically active and kinase-dead fusion proteins bound p21 and PCNA. In fact, although lower levels of D1K2(KD) were expressed due to infection at a lower multiplicity of infection (MOI), D1K2(KD) actually associated with higher levels of p21 and PCNA than D1K2. Rapamycin induced a partial decrease in p21 association with each fusion protein. However, some p21 remained associated with both fusion proteins, and rapamycin did not significantly alter PCNA association with either the active or the

kinase-dead fusion protein. Another mutant of the cyclin D1-Cdk2 fusion protein containing a point mutation in the cyclin D1 domain that prevents interaction between cyclin D1 and Cdk2 domains was also ineffective in reversing rapamycin action (data not shown). The results in Fig. 4A and B are consistent with the results in Fig. 3 indicating that the stabilization of cyclin D1/Cdk2/p21/PCNA complexes correlates with the ability of cyclin D1 overexpression to diminish rapamycin-induced cell cycle arrest. The results in Fig. 4 extend those observations by showing that stabilization of cyclin D1/Cdk2/p21/PCNA complexes alone is not sufficient to override rapamycin action but that these complexes must also possess kinase activity. Together, the results in Figs. 1, 2, 3, and 4 provide compelling evidence that cyclin/Cdk/p21/PCNA complexes are key targets of rapamycin action and that rapamycin may inhibit cancer cell proliferation largely through disruption of this class of cell cycle regulatory complexes. The results also imply that cyclin D1 reverses rapamycin action by increasing p21 levels and stabilizing catalytically active cyclin/Cdk/p21/PCNA kinase complexes (Fig. 3A) or by driving the formation of complexes containing Cdk2 and PCNA in the absence of p21 (Fig. 3B).

**Rapamycin derivative RAD001 inhibits tumor growth *in vivo*.** We next sought to determine whether rapamycin derivative RAD001 inhibits the growth of mouse mammary tumors *in vivo* through the same mechanisms as in our *in vitro* studies employing human mammary carcinoma cells. MMTV-*c-neu* transgenic mice were employed for these studies because the human homologue of *c-neu*, Her2, is overexpressed in ~30% of human breast cancers, making this model relevant to human breast cancer (26, 27). Initial studies were aimed at verifying that RAD001 inhibited mammary tumor growth in female MMTV-*c-neu* mice. In these experiments, mice were treated by oral gavage with either



**Figure 4.** Reversal of rapamycin-induced cell cycle arrest by a cyclin D1-Cdk2 fusion protein requires kinase activity. **A**, BT549 cells were left uninfected or infected with adenoviruses encoding GFP, the cyclin D1-Cdk2 fusion protein (Ad.D1K2), or a kinase-dead mutant of the cyclin D1-Cdk2 fusion protein [Ad.D1K2(KD)] at increasing MOIs (black triangles) of 460, 1,840, or 3,675 and incubated for 24 hours. The cells were fed with normal growth medium or medium containing 100 nmol/L rapamycin and incubated an additional 24 hours. The cells were subjected to [<sup>3</sup>H]thymidine incorporation assays as in Fig. 1. For each pair of control/rapamycin-treated samples, the values were normalized such that the control value was 100%. \*,  $P < 0.05$ , the rapamycin sensitivity of cells expressing the cyclin D1-Cdk2 fusion protein is significantly different from that of cells expressing the kinase-dead cyclin D1-Cdk2 fusion protein, GFP, or uninfected cells. **B**, BT549 cells were treated with Ad.GFP, Ad.D1K2, or Ad.D1K2(KD) at a MOI of 200, 200, or 100, respectively, and incubated for 24 hours. The cells were treated for an additional 24 hours with or without 100 nmol/L rapamycin. Cell extracts were prepared and subjected to direct immunoblot analysis (right) or incubated with anti-Flag-agarose to purify the Flag-tagged cyclin D1-Cdk2 and cyclin D1-Cdk2(KD) fusion proteins and associated proteins (left). HIRA was used as a loading control.



**Figure 5.** RAD001 inhibits the growth of tumors in MMTV-*c-neu* transgenic mice and blocks the formation of cyclin D1/Cdk2 complexes in tumors. **A**, tumor-bearing MMTV-*c-neu* mice were treated with vehicle or 2.5 mg/kg RAD001 for successive cycles of 5 consecutive treatment days followed by 2 days with no treatment. *Veh-1*, *Veh-2*, *Veh-3*, *Veh-4*, and *Veh-5* and *RAD-1*, *RAD-2*, *RAD-3*, *RAD-4*, and *RAD-5*, tumor growth curves for individual mice treated with the vehicle control or RAD001, respectively. During treatment, tumors were measured and tumor volumes were calculated. Data are plotted as tumor volume (cm<sup>3</sup>) versus the number of treatment days. \*During the treatment regimen, all of the vehicle mice formed secondary tumors, whereas secondary tumors were not observed in the RAD001-treated animals. **B**, tumor extracts were prepared and subjected to immunoprecipitation (IP) with antibodies to Cdk2 or preimmune rabbit IgG as a control followed by immunoblot analysis (top). The supernatants from the immunoprecipitation were retained and subjected to direct immunoblot analysis (bottom).

RAD001 or the vehicle control and tumor dimensions were measured over time. Figure 5A shows that the growth rate of tumors in vehicle-treated mice were significantly steeper than those observed in the RAD001-treated animals. To quantitate the results, the growth curve for each mouse was fitted to a line using least-squares analysis, allowing the calculation of a slope of the growth curve for each mouse. The average growth rate of tumors in the vehicle-treated mice ( $n = 5$ ) was 34 mm<sup>3</sup>/d, and the average growth rate of tumors in the RAD001-treated mice ( $n = 5$ ) was -2.2 mm<sup>3</sup>/d. The differences between the mean tumor growth rates of the vehicle- and RAD001-treated mice were statistically significant ( $P = 0.002$ ). It should also be noted that all vehicle-treated mice formed secondary tumors during treatment, whereas none of the RAD001-treated mice exhibited secondary tumors. Immunoblot analyses of tumor lysates indicated that RAD001 potentially inhibited the phosphorylation of the mTOR substrates 4EBP1 and p70<sup>s6k</sup> in the tumors *in vivo* (data not shown).

**RAD001 disrupts Cdk2/cyclin D1 complexes in mouse mammary tumors *in vivo*.** We hypothesized that RAD001 inhibition of tumor growth resulted from the disruption of cyclin/Cdk/p21/PCNA complexes as observed in cell culture experiments (Fig. 3A). To test this hypothesis, tumor extracts from vehicle- or RAD001-treated mice were subjected by immunoprecipitation studies using Cdk2 antibodies. The results were analyzed by immunoblot of both the immunoprecipitates and the proteins remaining in the supernatants (Fig. 5B). Unfortunately, we were unable to detect p21 or PCNA in either the immunoprecipitates or the supernatants. This is not unexpected based on the high sensitivity of MMTV-*c-neu* tumors to RAD001 and our *in vitro* studies (Fig. 2B) showing that cells with low or undetectable p21 levels (e.g., MDA-MB-361 and MCF10A) exhibit the highest

rapamycin sensitivity. Strikingly, RAD001 decreased the levels of cyclin D1 associated with Cdk2, although the levels of cyclin D1 in the supernatants from the RAD001-treated tumors were no different than the levels observed in the supernatants from the vehicle-treated tumors. Similar results were obtained with a total of three matched pairs of vehicle- and RAD001-treated mice. These results suggest that RAD001 prevented the assembly of cyclin D1/Cdk2 complexes in the tumors. Although p21 has been suggested to be an assembly factor for cyclin/Cdk complexes (9, 19, 28, 29) without being able to detect p21 levels in the tumors, we cannot formally rule out the possibility that RAD001 prevents the formation of cyclin D1/Cdk2 complexes independently of effects on p21 levels. However, the disruption of cyclin D1/Cdk2 complexes by RAD001 *in vivo* is consistent with the possibility that p21 is down-regulated by RAD001 in the tumors because the levels of cyclin D1/Cdk2 complexes are decreased in p21-deficient cells relative to the parental cells (Fig. 3B). Regardless of the mechanism by which RAD001 prevents the formation of cyclin D1/Cdk2 complexes, these results are consistent with the results in Figs. 3 and 4 showing that rapamycin induces cell cycle arrest by disrupting cyclin D1/Cdk2/p21/PCNA complexes. It was shown previously that rapamycin disrupts the formation of cyclin/Cdk complexes in cells in culture (30), but to our knowledge this is the first demonstration that rapamycin derivatives might function by disrupting cyclin/Cdk complexes in tumors *in vivo*.

## Discussion

**Cdk2/PCNA complexes are targets of rapamycin action.** Rapamycin was shown to prevent the formation of cyclin D1/Cdk complexes some time ago, but the molecular basis for this was

unknown (30). Here, we show that the disruption of cyclin D1/Cdk2/p21/PCNA complexes correlates with rapamycin-induced cell cycle arrest and that this disruption correlates best with down-regulation of cellular p21 levels. This result is in agreement with previous findings that p21 facilitates the formation of complexes containing Cdk2 and PCNA (9).

The observation that decreases in the levels of the Cdk inhibitor p21 correlates with rapamycin-induced cell cycle arrest may seem contradictory; however, there are ample data indicating that p21 can in some cases promote cell proliferation. Tumors driven by Wnt-1 overexpression were observed to grow more rapidly in a p21<sup>+/-</sup> background than in either a p21<sup>-/-</sup> or a p21<sup>+/+</sup> background (31). In fact, in these studies, approximately twice as much cyclin D1-associated Rb kinase activity was observed in the p21<sup>+/-</sup> tumors relative to tumors in a p21<sup>-/-</sup> or p21<sup>+/+</sup> background. These results suggest that an optimal basal level of p21 exists and that either a higher or a lower level of p21 inhibits cell proliferation. Consistent with this notion, p21 stimulates the kinase activity of cyclin/Cdk complexes *in vitro* at low concentrations but potently inhibits kinase activity at higher concentrations (19, 28). Additionally, many growth factors increase p21 expression (32, 33), and the *p21* gene is rarely mutated in cancers (34). These reports suggest that an important function of p21 is to serve as an assembly factor for Cdk complexes.

p21 and p27 have been suggested to serve as assembly factors for Cdk complexes (29). Rapamycin down-regulates p21 levels (Fig. 2; refs. 3, 17, 35); however, we have not observed any effect of rapamycin on the levels of p27 in any of the cancer cell lines that we have analyzed (data not shown). In other cell types, such as T cells, rapamycin increases p27 levels (36, 37). This would suggest that rapamycin selectively disrupts Cdk complexes that require p21 as an assembly factor but not those that require p27 as an assembly factor. Rb family members p130 and p107 were shown to bind to cyclin E/Cdk2 complexes in p21- and p27-deficient cells and to regulate the activity of these complexes (38–40). It is unknown whether p130 and p107 serve as assembly factors for a subset of cyclin/Cdk complexes in the absence of p21 and p27. In any case, we have been unable to detect the presence of p130 or p107 in association with cyclin D1/Cdk2 complexes, although cyclin E/Cdk2 complexes isolated under identical conditions contain abundant levels of both p130 and p107 (data not shown). We reported previously that cyclin D1/Cdk4 complexes preferentially bind p27, whereas cyclin D1/Cdk2 complexes preferentially bind p21 (9). Taken together, these data might indicate that cyclin D1/Cdk2 complexes are inactivated by rapamycin treatment in a relatively selective manner through p21 down-regulation. The cyclin D1-Cdk2 fusion protein may be effective in reversing rapamycin-mediated cell cycle arrest through the stabilization of p21 levels as observed with overexpression of cyclin D1 alone. It is also possible that because of the physical linkage of the cyclin D1 and Cdk2 domains together the cyclin D1-Cdk2 fusion protein does not require p21-dependent assembly function.

The results of studies with p21-deficient mice indicate that p21 is not required for organismal development, but this does not rule out the possibility that low levels of p21 are required for optimal tumor cell proliferation. For example, deletion of Cdk2, Cdk4, and Cdk6 or cyclins D1, D2, and D3 does not prevent normal development or abrogate cell proliferation in mice but renders cells from these mice significantly more resistant to immortalization or oncogenic transformation (41–44).

It has been suggested that PCNA serves as an adaptor to bring together Cdk2 and PCNA-associated Cdk2 substrates (45). Our

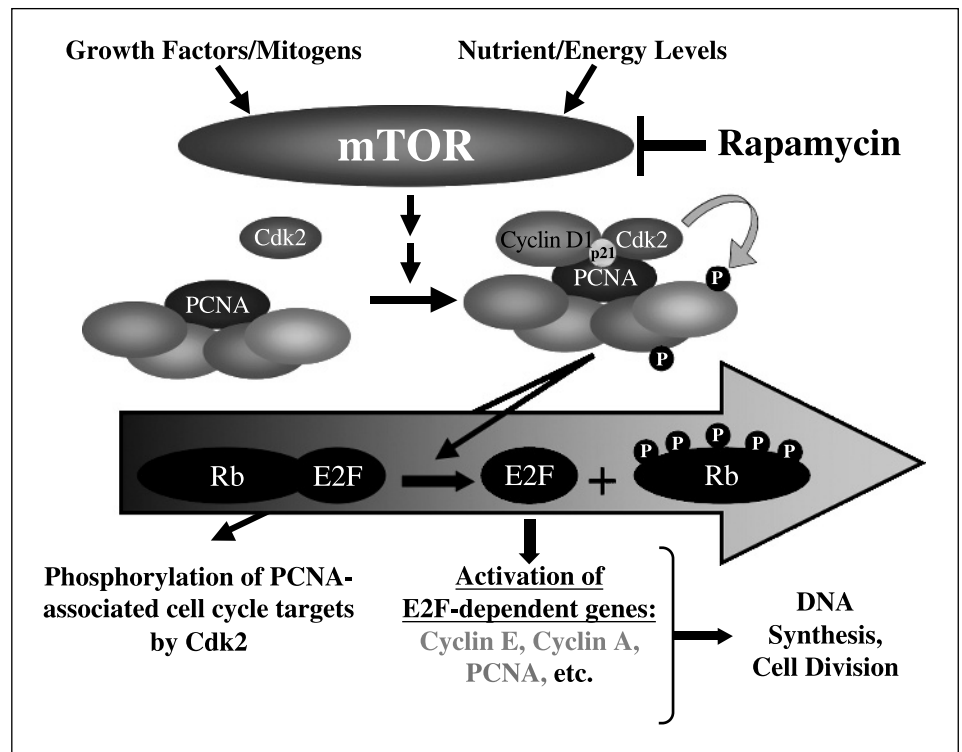
observation that p21 facilitates the formation of complexes between PCNA and Cdk2 implies that if this hypothesis is correct then rapamycin down-regulation of p21 abrogates the adaptor function of PCNA and prevents the Cdk2-dependent phosphorylation of this select group of substrates. This hypothesis is supported by the finding that the formation of complexes between a kinase-dead cyclin D1-Cdk2 fusion with p21 and PCNA is not sufficient to reverse rapamycin-induced cell cycle arrest but that the presence of associated kinase activity is also required. The finding that rapamycin still partially decreased p21 association with the cyclin D1-Cdk2 fusion protein without altering the level of associated PCNA may indicate that native cyclin D1/Cdk2 complexes require p21 association to observe PCNA binding to the complex, whereas the cyclin D1-Cdk2 fusion protein does not. This possibility is supported by reports showing direct interactions between cyclin D1 and PCNA (46) and Cdk2 and PCNA (45).

Together, our results are most consistent with the model in Fig. 6 that mTOR is activated by growth factors, high nutrient levels, and high energy levels (reviewed in refs. 47–49) leading to increased cellular p21 and cyclin D1 levels. p21 facilitates the assembly of cyclin/Cdk/p21/PCNA complexes. Cyclin/Cdk/p21/PCNA complexes stabilize the expression of the cyclin and p21 components and catalyze the phosphorylation of cellular substrates. Rapamycin inhibits mTOR activity, preventing the assembly of these complexes and destabilizing p21 and cyclin D1. This model predicts that rapamycin may not strongly inhibit the phosphorylation of Rb (e.g., Fig. 2) because Rb phosphorylation can be carried out by many different Cdk complexes but rather that rapamycin will most strongly inhibit the phosphorylation of Cdk2 substrates that require p21- or PCNA-dependent adaptor function for their phosphorylation. Rigorous proof of this model will require the identification of bona fide cellular substrates that meet these criteria and the demonstration that rapamycin blocks the Cdk2-dependent phosphorylation of these proteins.

**Cyclin D1 suppression of rapamycin-mediated cell cycle arrest correlates with stabilization of Cdk2/PCNA complexes.** Numerous reports have been published showing that in various cell lines rapamycin down-regulates the levels of cyclin D1 (1, 2, 16). Because of these results and the observation that cyclin D1 overexpression reverses rapamycin antiproliferative effects, it was suggested that cyclin D1 may be the primary target of rapamycin action (3). However, levels of Cdk2-associated p21 and PCNA correlate better with rapamycin-induced cell cycle arrest and cyclin D1 reversal of rapamycin-induced cell cycle arrest than with Cdk2-associated cyclin D1 levels (Figs. 1 and 3). A report by Huang et al. showed that rapamycin-induced cell cycle arrest was not observed in cells lacking p21 (50), suggesting that p21, rather than cyclin D1, is the more important target of rapamycin antiproliferative effects.

Nonetheless, the observation that cyclin D1 overexpression decreases rapamycin antiproliferative effects has important implications regarding the use of rapamycin derivatives as anticancer agents. Breast cancers, for example, exhibit cyclin D1 overexpression ~45% to 50% of the time (4, 5); therefore, assessing tumor cyclin D1 levels may aid in predicting which tumors are favorable targets for rapamycin treatment. Further, because rapamycin inhibition of cell proliferation *in vitro* and tumor growth *in vivo* both correlated with disruption of cyclin D1/Cdk2/p21/PCNA complexes, analysis of tumor biopsies or cells cultured from tumor biopsies for rapamycin-induced disruption of cyclin D1/Cdk2/p21/PCNA complexes may be useful in predicting tumor response. Our analyses of tumors from mice treated with vehicle or

**Figure 6.** Model for rapamycin regulation of the structure of cyclin/Cdk/p21/PCNA complexes. mTOR activity and the subsequent stimulation of downstream effector pathways is positively regulated by growth factors, high amino acid levels, and high energy charge. mTOR increases p21 and cyclin D1 levels. p21 facilitates the assembly of quaternary complexes containing cyclins, Cdks, p21, and PCNA. Cyclin/Cdk/p21/PCNA complexes catalyze Rb phosphorylation and may also specifically target PCNA-associated proteins for phosphorylation.



RAD001 indicate that it may be possible to examine the effect of rapamycin derivatives on the structure of cyclin D1/Cdk2/p21/PCNA complexes in human tumor biopsies. Rapamycin disruption of cyclin D1/Cdk2/p21/PCNA complexes might serve as a useful surrogate marker to monitor antitumor efficacy during treatment with rapamycin derivatives. In addition, p21, Cdk2, and PCNA have important roles in DNA damage checkpoint responses (51–53), suggesting that rapamycin may influence the response of tumors to radiation or DNA-damaging agents in combination therapies. In fact, a recent report showed that RAD001 sensitizes tumor cells to cisplatin-induced apoptosis by suppressing p21 translation and lowering cellular p21 levels (35).

In summary, we presented previously data supporting the hypothesis that cyclin D1/Cdk2 complexes contribute to cyclin D1-mediated cell transformation (9). We now extend this

hypothesis to suggest that, collectively, cyclin/Cdk/p21/PCNA complexes are an important drug target and are disrupted and functionally inactivated by rapamycin, rapamycin derivatives, and possibly other inhibitors of the mTOR/p70<sup>s6k</sup> signaling pathway (16, 54, 55).

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