Proliferation of cancer cells despite CDK2 inhibition

Osamu Tetsu and Frank McCormick*

Cancer Research Institute and Comprehensive Cancer Center, University of California, San Francisco, California 94143
*Correspondence: mccormick@cc.ucsf.edu

Summary

We have investigated the contribution of CDK4 and CDK2 inhibition to G1 arrest in colon cancers following inhibition of the MEK/MAP kinase pathway. CDK4 inhibition is sufficient to cause arrest, but inhibition of CDK2 by p27 Kip1 redistribution or ectopic expression has no effect on proliferation. Likewise, inhibition of CDK2 through expression of dominant-negative (DN) CDK2 or antisense oligonucleotides did not prevent cell proliferation in these cells. We therefore tested whether CDK2 activity is dispensable in other cells. Surprisingly, osteosarcomas and Rb-negative cervical cancers continued to proliferate after depletion of CDK2 through antisense oligonucleotides or small interfering (si) RNA. Here we report of sustained cell proliferation in the absence of CDK2, and we suggest that CDK2 is not a suitable target for cancer therapy.

Introduction

Genetic alterations in the Rb pathway are a hallmark of cancer and have revealed a number of possible therapeutic targets, including the cyclin-dependent kinases CDK4 and CDK2 (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). CDK4 activity is clearly implicated in cancer by alterations in its cyclin D partner and its regulator p16 Ink4a, as well as mutations in CDK4 itself (Sherr and McCormick, 2002), whereas CDK2 is not affected directly by mutations or gene copy number changes that cause cancer (Sherr and Roberts, 1999). It is currently believed that progression through the cell cycle from G1 to S phase requires sequential activation of CDK4 and CDK2 (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). The role of CDK4 is well established: it phosphorylates the Rb protein and releases E2F activity (Reed, 1997). E2F in turn activates transcription of a number of genes involved in regulating and mediating DNA synthesis (Herrera et al., 1996). CDK4 activity is dispensable in cells lacking the Rb protein: many cancer cells, for example, fail to express Rb through sporadic mutation of the Rb gene, and these cells fail to growth arrest in the presence of CDK4 inhibitors (Lukas et al., 1995a, 1995b; Koh et al., 1995; Madema et al., 1995). On the other hand, the function of CDK2 is less clear. A dominant-negative (DN) form of CDK2 prevents growth of cells in culture (van den Heuvel and Harlow, 1993; Hu et al., 2001), and microinjection of antibodies against CDK2, cyclin E, or cyclin A block initiation of DNA synthesis in mammalian cells (Ohtsubo et al., 1995; Pagano et al., 1992; Tsai et al., 1993). Furthermore, expression of the CDK2 inhibitor p27 Kip1 generally causes growth arrest (Polyak et al., 1994; Toyoshima and Hunter, 1994; Kwon and Nordin, 1997; Dirks et al., 1997; Katayose et al., 1997; Blain et al., 1997; Cheng et al., 1999; Yang et al., 2001), although exceptions have been reported (Naruse et al., 2000). However, there is little consensus on the critical substrates of CDK2 (reviewed in Reed, 1997). Rb appears to be a target (Hinds et al., 1992), though the sites of phosphorylation have been controversial (Zarkowska and Mittnacht, 1997). Other substrates for CDK2 include the centrosome protein nucleophosmin and proteins involved in DNA replication (Okuda et al., 2000; Nigg, 2001; Stucke et al., 2002). However, it has not yet been proven that phosphorylation is necessary for proliferation of mammalian cells in culture. In *Xenopus* extracts, CDK2 is necessary for sustaining multiple rounds of DNA replication, but in the initial round, other kinases such as aurora kinase may be sufficient (Nigg, 2001). However, in mammalian somatic cells, CDK2 may be regulated differently and in cancer cells, aurora kinase and related enzymes are often misregulated or overexpressed (Reed, 1997; Bischoff et al., 1998; Nigg, 2001).

In this paper, we report that CDK2 activity is dispensable for cancer cell proliferation. We will discuss the possibility that high levels of CDK4 activity in these cells may compensate for requirement of CDK2 during cell cycle progression and also suggest that in Rb-minus tumor cells, both CDK4 and CDK2 may be unnecessary for proliferation.

Results

cyclin D1, cyclin D3, CDK4, and p21 Cip1 proteins are depleted by treatment of colon carcinoma cells with MEK inhibitors, resulting in complete loss of CDK4 activity

Inhibitors of the Ras/Raf/MEK/MAP kinase pathway are currently undergoing clinical evaluation, based on their ability to

SIGNIFICANCE

This paper reports of sustained cell proliferation in the absence of CDK2 activity. Cyclin E/CDK2 activity is frequently increased in tumors, but it is not clear that this is a cause or a consequence of the disease since there is little consensus on the biochemical targets of CDK2. Rb appears to be a target, but, as shown here, CDK4 is able to phosphorylate Rb even at CDK2 preferred phosphorylation sites. We propose that increased levels of CDK4 or E2F activity in cancer cells may compensate for the requirement for CDK2 activity. These results show that CDK2 is not a suitable target for treatment of cancers, and also question the role of CDK2 in cell proliferation.

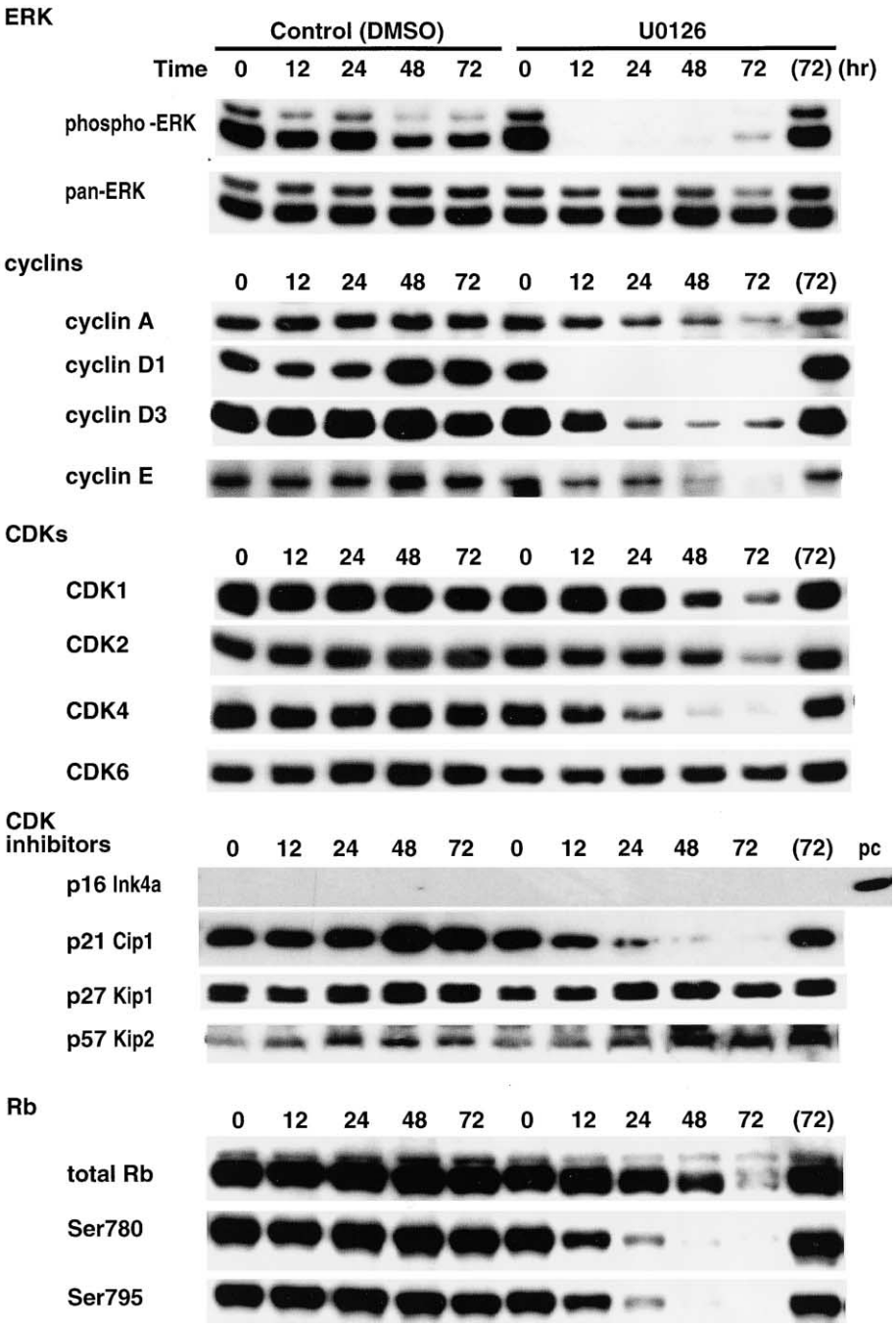

Figure 1. cyclin D1, cyclin D3, CDK4, and p21 Cip1 are strongly suppressed by the MEK inhibitor U0126 in colon carcinoma cells

Exponentially growing SW480 cells were treated with DMSO (Control) or 10 μM MEK inhibitor U0126. Cells were collected at the indicated times (0, 12, 24, 48, and 72 hr) after treatment. Seventy-two hours with parentheses () indicates that U0126 was withdrawn at 48 hr and collected at 72 hr to show that the effect is reversible. Western blots were performed. Phosphorylated (phospho)-ERK was completely suppressed by 10 μM MEK inhibitor U0126. Positive control (pc) from cell lysates of HeLa cells was used to detect p16 Ink4a expression. Phosphorylation status for the endogenous Rb expression was analyzed with total Rb antibody and Rb phosphorylation antibodies specific to CDK4 (6).

inhibit growth of cancer cells, particularly those derived from colon cancers. However, the mechanisms by which they mediate their effects are not clear (Sebolt-Leopold et al., 1999). We examined the mechanism of action of U0126 and PD 184352, two structurally unrelated compounds that selectively inhibit MEK1 and MEK2 (Favata et al., 1998; Sebolt-Leopold et al., 1999; Davies et al., 2000). First, we performed flow cytometric cell cycle analysis of various colon cancer cells after U0126 treatment. Cells accumulated in G1 within 12 hr of treatment with U0126 or PD 184352. No sub-G1 phase population was observed, indicating that cells did not undergo apoptosis (data not shown). The MAP kinase cascade is therefore a key signal transduction pathway for the G1/S transition in colon carcinoma cells (Sebolt-Leopold et al., 1999).

To address the molecular mechanism of G1 arrest following MEK inhibition, we analyzed expression levels of known cell cycle regulators (Figure 1). After 12 hr of treatment with U0126, when cells were growth arrested in G1, phosphorylated-ERK had disappeared, indicating that MEK activity was completely inhibited. We observed a dramatic reduction of cyclin D1, cyclin D3, CDK4, and p21 Cip1 during this period of exposure to U0126. After 48 hr, cyclin A, cyclin E, and CDK1 (CDC2) levels had also decreased, but CDK2 expression was relatively stable. Over this time course, no difference was observed in expression

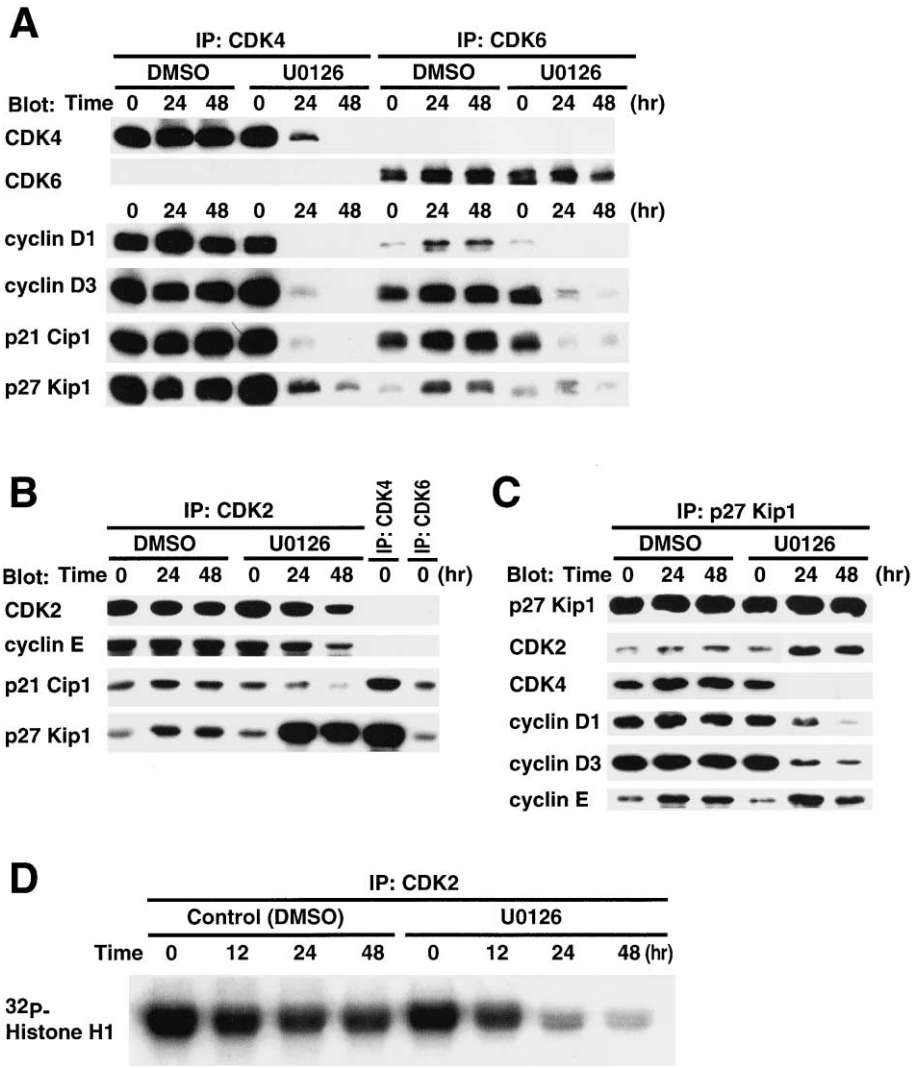

Figure 2. p27 Kip1 redistribution from CDK4 to CDK2 and CDK2 kinase inhibition by U0126 in SW480 cells

A–C: Immunoprecipitation and Immunoblotting analysis. Cells were collected at indicated time points (0, 24, and 48 hr). Protein was precipitated with rabbit antibodies (2 μg for each reaction) specific to CDK2, 4, 6, or p27 Kip1, respectively. Immune complexes were resuspended and subjected to SDS-PAGE. Immunoblotting was performed using indicated mouse monoclonal antibodies.

A: Samples precipitated with CDK4 or CDK6 antibody were loaded onto the same gel.

B: Dissociated p27 Kip1was redistributed from CDK4 and 6 to CDK2 by U0126.

C: p27 Kip1 redistribution from CDK4 to CDK2 was shown on the same blot by using p27 Kip1 antibody for immunoprecipitation.

D: CDK2-associated Histone H1 kinase assay.

of CDK6 or p27 Kip1. p16 Ink4a was not expressed in these cells, probably because its promoter is hyper-methylated (Burri et al., 2001). Cyclin D2 was not detected in SW480, SW48, DLD-1, and HCT 116 colon cancer cells (data not shown). Similar results were obtained in SW480 cells with PD 184352 (data not shown). Growth arrest may therefore be caused by downregulation of either cyclin D1, cyclin D3, CDK4, or p21 Cip1 since levels of these proteins decreased within the 12 hr time period in which G1 arrest occurred. All of these molecules are essential for active complexes of cyclin D/CDK4. Inhibition of CDK4 kinase activity was therefore expected (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). To confirm this, we tested the phosphorylation status of the endogenous Rb protein particularly for CDK4 (6) phosphorylation sites, Ser780 and Ser795 (Kitagawa et al., 1996; Brugarolas et al., 1999). CDK4 phosphorylation sites on Rb were strongly inhibited by inhibition of MEK activity. These effects preceded changes in the level of total Rb itself (see 12, 24, and 48 hr). U0126 was withdrawn after 48 hr and replaced with fresh medium for a further 24 hr. These samples are referred to as (72 hr) in the figure; clearly the effects of U0126 on these cell cycle regulators are completely reversible.

Redistribution of p27 Kip1 from CDK4 to CDK2 following loss of cyclinD/CDK4, resulting in inhibition of CDK2

Redistribution of Cip/Kip family members following increases in expression of Ink4 (inhibitors of CDK4) proteins has been described previously (Reynisdottir and Massague, 1997; Jiang et al., 1998; McConnell et al., 1999; Swarbrick et al., 2000). More recently, it was reported that rapid proteolysis of cyclin D1 leads to release of p21Cip1 from CDK4 to inhibit CDK2 activity (Agami and Bernards, 2000). Binding of displaced Cip/Kip proteins to CDK2 and consequent inhibition of CDK2 kinase activity are thought to contribute to growth arrest mediated by these inhibitors. A recent report from analysis of CDK4 knockout mice suggests a model for p27 Kip1 redistribution (Tsutsui et al., 1999). Furthermore, overexpression of cyclin D1 and cyclin D2 resulted in sequestration of p21 Cip1 and p27 Kip1 (Perez-Roger et al., 1999; Bouchard et al., 1999). These observations suggest that loss of expression of cyclin D/CDK4 complexes results in redistribution of p27 Kip1 to inhibit CDK2 kinase activity. To address the possibility that p27 Kip1 was redistributed from CDK4 and 6 to CDK2 following MEK inhibition, we per-

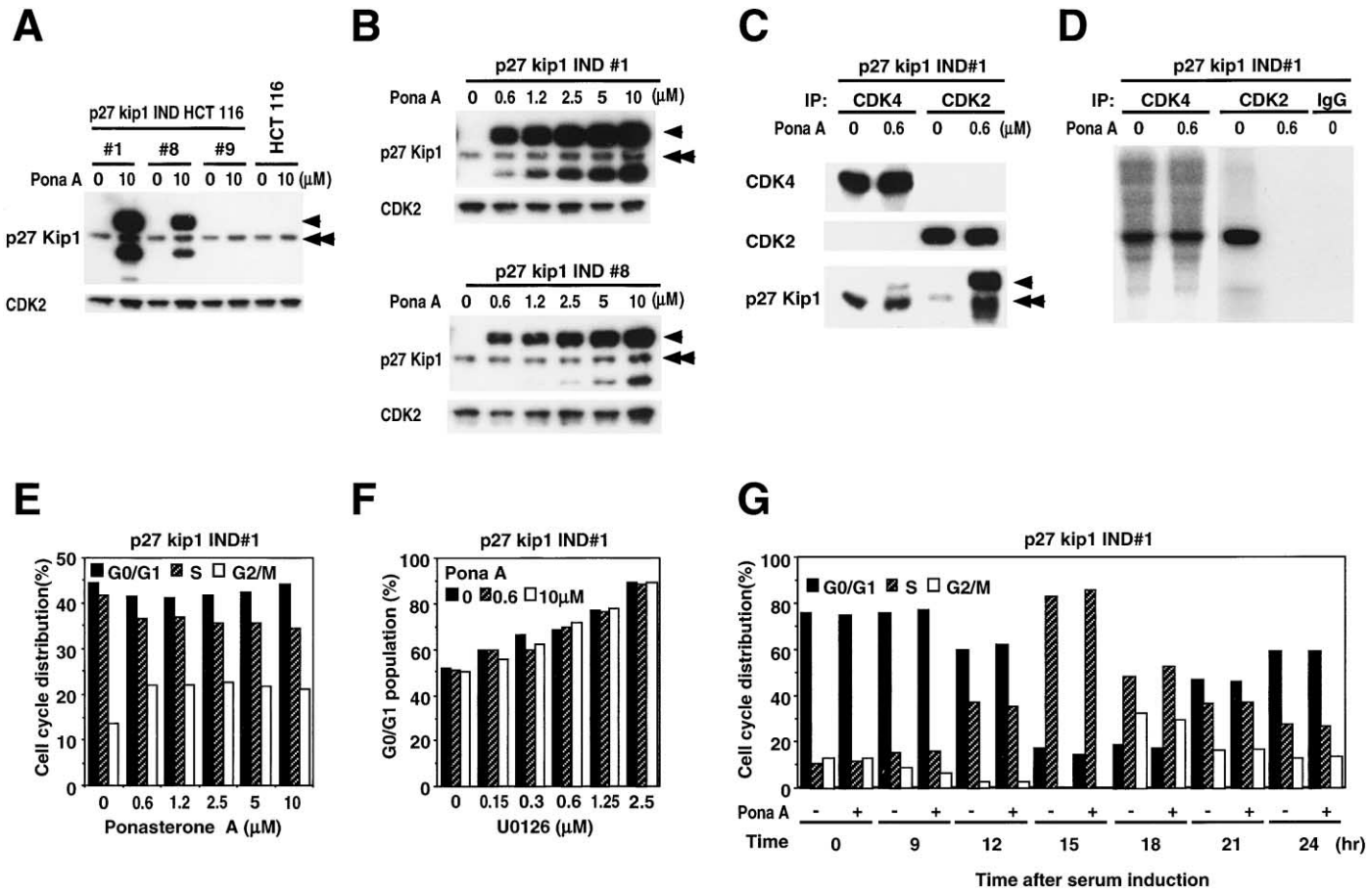

Figure 3. p27 Kip1 does not cause the G1 arrest in HCT 116 colon cancer cells

Samples were collected at 24 hr after the simultaneous induction with Ponasterone A (**A–F**) and U0126 (**F**). Indicated concentration of Ponasterone A (Pona A) was used for the gene expression (**A**–**F** and **H**). Immunoblotting was performed (**A** and **B**).

A: p27 kip1 ecdysone-inducible (IND) HCT 116 cell lines. Parental HCT 116 cells show the level of endogenous protein.

B: Dose-dependent induction of p27 Kip1 ectopic expression. Double arrow denotes Myc-27. Single arrow denotes endogenous p27 Kip1.

C: Immunoprecipitation and Immunoblotting on p27 Kip1 overexpression in HCT 116 cells.

D: CDK4-associated Rb kinase assay and CDK2-associated histone H1 kinase assay on p27 Kip1-overexpressed HCT 116 cells. Samples were coprecipitated either with CDK4, CDK2, or control IgG antibodies, respectively.

E–**G:** Flow cytometric cell cycle analysis.

E and **G:** Cell cycle distribution after p27 Kip1 induction in HCT 116 cells

F: Combination of U0126 and p27 Kip1 had no effect on the cell cycle profile.

G: p27 kip1 IND HCT 116 line #1 cells were serum starved for 48 hr in the absence (−) or presence (+) of 10 μM Ponasterone A and then stimulated by the addition of 10% FBS containing media with or without 10 μM Ponasterone A to allow synchronous progression. Samples were collected at indicated time points.

formed immunoprecipitation and immunoblotting analysis on CDKs to look for associated regulatory molecules (Figures 2A–2C). In panel A, CDK4 or CDK6 coprecipitated samples were analyzed on the same blot. As expected from Figure 1, cyclin D1, cyclin D3, and p21Cip1 were not associated with CDK4 or 6 after 24 hr because of reduced expression (Figure 2A). CDK6 expression was not affected significantly by U0126, but reduced association of p27 Kip1 with CDK6 suggested that p27 Kip1 could not bind to CDK6 in the absence of MEK/MAPK activity. p27 Kip1 completely disappeared from CDK4 and 6 after exposure to U0126, although total p27 Kip1 expression was not changed (Figure 1). An apparent preference of cyclin D1 and p27 for CDK4 relative to CDK6, in contrast to the behavior of cyclin D3 and p21, enhanced this effect (Figure 2A). We also performed immunoprecipitation with antibodies against CDK2 to test association with p27 Kip1. There was a significant increase in association of p27 Kip1 with CDK2 after treatment with U0126 (Figure 2B). This observation was confirmed by immunoprecipitation with p27 Kip1. Redistribution of p27 Kip1 from CDK4 to CDK2 with U0126 is shown on the same blot (Figure 2C). Almost identical results were obtained from HCT 116 cells (data not shown). To investigate the effects of redistribution of p27 Kip1 to CDK2, we tested CDK2-associated Histone H1 kinase assay following U0126 treatment (Figure 2D). After 24 hr, CDK2 activity was strongly inhibited by U0126. We conclude that CDK4 activity was inhibited through loss of expression in cyclin D1, cyclin D3, CDK4, and p21 Cip1. CDK2 activity was inhibited through redistribution of p27 Kip1 (summarized in Figure 9).

Overexpression of p27 Kip1 does not cause growth arrest in colon cancers

To establish the relative importance of inhibiting CDK4 and CDK2 activity, we generated a p27 Kip 1 ecdysone-inducible system in HCT 116 colon carcinoma cells (Figures 3A–3D). Overexpression of p27 Kip1 induces G1 arrest by altering CDK2 activity in Mv1Lu mink epithelium, Saos-2, HeLa, mouse embryo fibroblasts, U343 astrocytomas, MDA-MB-231 breast cancer cells, H322, A549, and SQ-5 lung cancer cells (Polyak et al., 1994; Toyoshima and Hunter, 1994; Kwon and Nordin, 1997; Dirks et al., 1997; Katayose et al., 1997; Blain et al., 1997; Cheng et al., 1999; Yang et al., 2001), though no effect was seen in other cancer cells, such as H69 and Lu-135 lung cancer cells (Naruse et al., 2000). We expected that overexpression of p27 Kip1 would cause G1 arrest in colon cancers without addition of U0126. We generated two cell lines (#1 and #8) in which p27 Kip1 was highly inducible (Figure 3A). Ectopic expression was distinguished from endogenous expression by the reduced mobility of the c-*myc* epitope tagged p27 Kip1 protein. Analysis of a nonexpressing line (#9) and parental cells shows that the endogenous p27 Kip1 expression was not affected by the ecdysone analog Ponasterone A (Figures 3A and 3B). Panel B shows that ectopic expression of p27 Kip1 was induced by Ponasterone A in a dose-dependent manner. To test whether ectopic expression of p27 Kip1 was functional, we performed immunoprecipitation and immunoblotting analysis and kinase assay on both CDK 4 and CDK2 (Figures 3C and 3D). Ectopically expressed p27 Kip1 was bound to both CDK4 and CDK2, but the majority associated with CDK2. Panel D shows that induced expression of p27 Kip1 caused a similar severe inhibition of CDK2 activity, as seen following treatment with U0126; in the same samples, CDK4 kinase activity was not affected. These results are in agreement with previous reports cited above and indicate that ectopic expression of p27 Kip 1 in HCT 116 cells was functional and comparable in activity to endogenous p27 Kip1 shown in Figures 3A–3D. In addition, expression of myc-tagged p27 Kip1 did cause growth arrest in U-2 OS osteosarcoma, T98G glioblastoma, and C33A cervical carcinoma cells as described below (Figure 6A).

We analyzed the cell cycle profile 24 hr after induction of p27 Kip1 (Figure 3E). Surprisingly, ectopic expression of p27 Kip1 did not cause growth arrest: no accumulation in G0/G1 and sub-G1 phase population was observed, although we detected a slight change in S and G2/M phases. Furthermore, there was no difference in cell cycle profile among cells expressing different levels of p27 Kip1 induced by Ponasterone A. Very similar results were obtained from another line, #8 induced with Ponasterone A for 24 or 48 hr (data not shown). To determine whether overexpression of p27 Kip1 had any effects on cell cycle progression from quiescence, p27 kip1 IND HCT 116 line #1 cells were serum starved for 48 hr in the presence or absence of Ponasterone A and then stimulated by the addition of 10% FBS containing media with or without Ponasterone A to allow synchronous progression (Figure 3G). Overexpression of p27 Kip1 did not show any delay throughout a complete cell cycle, suggesting that CDK2 is not necessary in the initial round of the cycle. We therefore tested the possibility that CDK2 is not necessary for sustaining multiple rounds of DNA replication. Viable cell numbers were counted for a week (Figure 4A) and a colony-forming assay was performed (Figure 4B). No differences were observed in the number of viable cells after p27 Kip 1 induction (Figure 4A). In the colony-forming assay, cells expressed ectopic p27 Kip1 for 2 weeks, and colonies were stained with crystal violet (Figure 4B). The number and size of colonies formed in the presence of ectopic expression of p27 Kip1 were similar to control. These data strongly suggest that colon cancer cells proliferate without CDK2 activity and that redistribution of p27 Kip1 to CDK2 following MEK inhibition is dispensable for G1 arrest. This was confirmed by combining U0126 with p27 Kip1 induction (Figure 3F). U0126 induced G1 arrest in a dose-dependent manner as mentioned above. However, no enhancement was seen by p27 Kip1 expression. Similar results were obtained from another line, #8 (data not shown). Thus, MEK inhibitors do not require redistribution of p27 Kip1 to cause G1 arrest in colon cancer cells. Indeed U0126 induced G1 arrest at 12 hr without altering CDK2 activity in growing SW480 colon cancer cells (Figure 2D). To address whether CDK4 kinase inhibition is sufficient to induce G1 arrest in colon cancer cells, we used a CDK4 inhibitor AG12275 to block CDK4 kinase activity (Toogood, 2001; Figures 4C and 4D). We used AG12275 rather than p16 Ink4a to inhibit CDK4 because small molecule CDK4 inhibitors are thought to inhibit kinase activities without changing assembly of cyclin D/CDK4 complexes (see Buolamwini, 2000 for review of CDK inhibitors). First, we evaluated the selectivity of CDK4 kinase inhibition. Figure 4C shows that AG12275 severely inhibited CDK4 activity; in the same samples, CDK2 kinase activity was not affected. AG12275 has been demonstrated to inhibit growth of HCT 116 colon carcinoma in a mouse xenograft model (Toogood, 2001). This was confirmed by flow cytometric cell cycle analysis with cultured cells (Figure 4D). To test whether p27 Kip1 redistribution was dispensable for growth arrest, we treated cells by combining AG12275 with p27 Kip1 induction. AG12275 induced G1 arrest in a dose-dependent manner; however, little enhancement was seen by p27 Kip1 expression, suggesting that partial inhibition of CDK4 did not render these cells sensitive to CDK2 inhibition. Thus, inhibition of CDK4 kinase activity but not CDK2 is responsible for G1 arrest in colon cancer cells, and CDK2 activity in these cells is not sufficient to support S phase entry in the absence of CDK4 activity.

p27 Kip1 expression is not necessary for growth inhibition by MEK inhibitors

To investigate the possibility that p27 Kip1 is dispensable for G1 arrest in these colon carcinoma cells, we used p27 kip1 antisense (AS) oligonucleotides to block p27 Kip1 protein production and then assessed the ability of U0126 to cause a G1 arrest in the setting of diminished p27 Kip1 protein level. First, we evaluated levels of p27 Kip1 protein after the AS oligonucleotides transfections. Growing SW480 cells were transfected either with mismatch (MM) oligonucleotide or p27 kip1 AS oligonucleotide (Figure 4E). p27 kip1 AS oligonucleotide but not MM oligonucleotide reduced levels of p27 Kip1 protein more than 95%. The effect was specific as the similar protein p21 Cip1 was not affected. To determine effects on cell growth, flow cytometric cell cycle analysis was performed. Twenty-four hours after the transfection with either MM oligonucleotide or p27 kip1 AS oligonucleotide, SW480 cells were synchronized to G1 phase by serum starvation for overnight and then stimulated by the addition for the next 12 hr of 10% FBS containing media with or without U0126 (Figures 4F–4H). The effect was significant, as MAPK activity was inhibited by U0126, and p27 kip1 AS

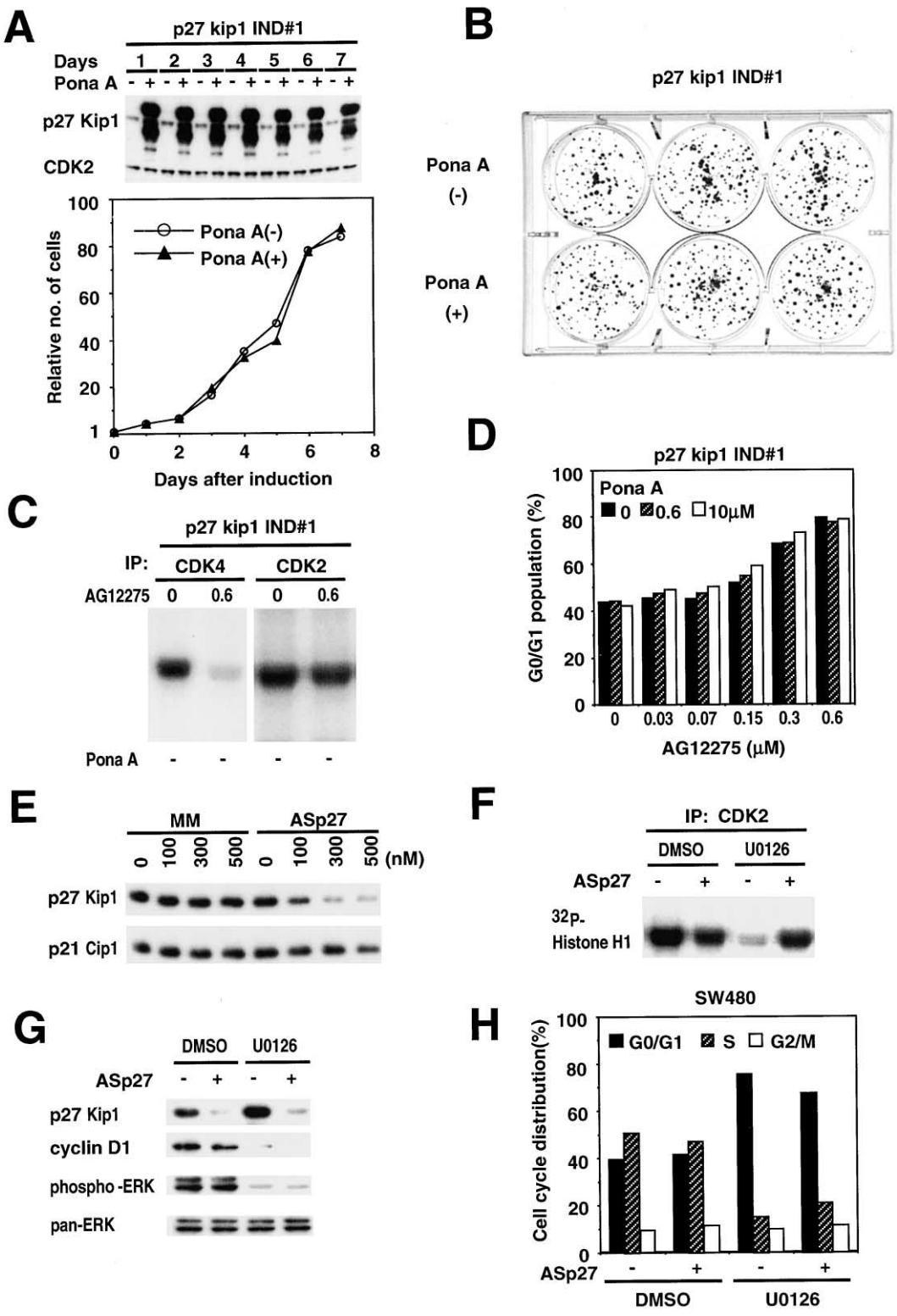

Figure 4. p27 Kip1 expression is not necessary for growth inhibition in colon cancer cells

Ten micromolar Ponasterone A (Pona A) was used for gene expression (**A**). Immunoblotting was performed (**A**). Samples were collected at 24 hr after the simultaneous induction with Ponasterone A (**D**) and AG12275 (**C** and **D**).

A: Viable cell number from p27 kip1 IND HCT 116 line #1 cells after Ponasterone A inducition. Cells cultured without (−) or with (+) 10 μM Ponasterone A were collected at the indicated days (0, 1, 2, 3, 4, 5, 6, 7 days) after induction and stained with trypan blue. Cell number was counted with a haemocytometer. During the time course, cells were continuously producing high levels of p27 Kip1 proteins (see the above Western blot).

B: Colony formation assay on p27 kip1 IND HCT 116 line #1 cells in the absence (−) or presence (+) of 10 μM Ponasterone A. One hundred single cells from p27 kip1 IND HCT 116 line #1 were cultured in the absence (−) or presence (+) of Ponasterone A. Fourteen days after induction, cells were stained with 0.5% crystal violet containing 20% ethanol. Tripricate cell cultures are shown.

oligonucleotide resulted in recovery of CDK2 kinase activity (Figures 4F and 4G). As we expected, p27 kip1 AS oligonucleotide did not restore growth arrest significantly, although a slight restoration was observed (Figure 4H): U0126 induced a growth arrest both in the MM oligonucleotide- and p27 kip1 AS oligonucleotide-transfected cell cultures. Similar data were obtained from the samples treated with U0126 for 24 hr in SW480 and HCT 116 cells (data not shown). These results indicate that p27 Kip1 expression was not responsible for growth inhibition by MEK inhibitors, and that restoration of CDK2 activity was not sufficient to overcome the effects of MEK inhibition.

A dominant-negative form of CDK2 does not cause growth arrest in colon cancer cells

To confirm that CDK2 kinase inhibition does not cause growth arrest in colon cancer cells, we established a D145N DN form of CDK2 in an ecdysone-inducible system. Overexpression of a DN CDK2 has been reported previously to induce G1 arrest in U-2 OS and Saos-2 osteosarcomas, T98G glioblastoma, and C33A cervical carcinoma cells (van den Heuvel and Harlow, 1993; Hu, et al., 2001). This protein is thought to inhibit endogenous CDK2 activity by sequestering cyclin E. We generated four SW480 cell lines (#24, #32, #34, and #43) in which DN CDK2 was highly inducible, out of 50 drug-resistant colonies that we tested (Figure 5A). Similar to the p27 Kip1-inducible system, ectopic expression was distinguished from endogenous expression by the reduced mobility of the HA epitope-tagged DN CDK2 protein. Figure 5A shows that ectopic expression of DN CDK2 was inducible by Ponasterone A. Induced expression of DN CDK2 severely inhibited CDK2 activity (Figure 5B), in agreement with previous reports (van den Heuvel and Harlow, 1993). However, ectopic expression of DN CDK2 did not prevent progression through the cell cycle after release from serum starvation, although a delay in S phase entry was observed (Figure 5C). Likewise, dose-dependent induction of DN CDK2 had little effect on the cell cycle profile of proliferating SW480 cells (clone #24), though a decrease in S phase and an increase in G0/G1 was detected (Figure 5D). Very similar results were obtained from another line, #32 (data not shown). These data strongly support the suggestion that CDK2 inhibition does not cause growth arrest in human colon cancer cells. Furthermore, partial inhibition of CDK4 by AG12275 did not render SW480 cells sensitive to CDK2 inhibition (Figure 5E). Similar results were obtained from another line, #32 (data not shown). Thus, CDK2 kinase activity is dispensable for G1/S transition in colon carcinoma cells.

To confirm that the DN CDK2 construct used in these experiments functions as described in previous reports (van den Heuvel and Harlow, 1993), we transfected U-2 OS, T98G, and C33A cells with DN CDK2. Significant increases in G0/G1 in U-2 OS and T98G cells were detected, as previously reported. C33A cells were slightly affected, but HCT 116 and SW480 colon cancer cells showed no effect. Myc-tagged p27 also caused arrest in U-2 OS, T98G, and C33A cells (Figure 6A). To address whether CDK4 kinase inhibition is sufficient to induce G1 arrest in colon cancer cells in this system, two forms of DN cdk4 expression vector were tested in HCT 116 and SW480 colon cancers and then we evaluated the effect by flow cytometric cell cycle analysis (Figure 6B). One encodes D158N cdk4, an analogous form of D145N cdk2. The other encodes T172A cdk4, in which the activating threonine residue at position 172 in the T loop is replaced with alanine (Kato et al., 1994a, 1994b; Matsuoka et al., 1994). The latter form binds cyclin D1 efficiently but does not phosphorylate the Rb protein (Coleman et al., 1997). T160A cdk2 corresponds to the T172A mutant of cdk4. Significant increases in G0/G1 were detected with either p16 ink4a or T172A DN cdk4 transfection, but other DN forms did not show any effect. The effects of DN T172A cdk4 and p16 ink4a confirm the observation using AG12275 that inhibition of CDK4 kinase activity but not CDK2 is responsible for G1 arrest in colon cancer cells.

Cancer cells proliferate despite CDK2 inhibition

To confirm that CDK2 kinase activity is dispensable in colon cancers, we directly inhibited CDK2 kinase activity by depletion of CDK2 expression through antisense (AS) oligonucleotides and then assessed the effect on cell cycle profile (Figures 7A and 7B). First, we evaluated levels of CDK2 protein after the AS oligonucleotide transfections. Growing SW480, HT-29, SW48, and HCT 116 colon cancer cells were transfected either with mismatch (MM) oligonucleotide or cdk2 AS oligonucleotide (Figure 7A). cdk2 AS oligonucleotide but not MM oligonucleotide significantly reduced levels of CDK2 protein. This was confirmed by Histone H1-associated CDK2 kinase assay. To determine effects on cell growth, flow cytometric cell cycle analysis was performed after 48 hr from the transfection with either MM oligonucleotide or cdk2 AS oligonucleotide. As we expected, depletion of CDK2 expression did not cause growth arrest: no accumulation in G0/G1 and sub-G1 phase population was observed. We conclude that CDK4 (6) activity is critical to colon cancer cell proliferation, but CDK2 activity is not. We therefore tested that CDK2 activity is dispensable in other cells (Figures 7C and 7D). Four cancer cell lines were tested: U-2 OS osteosarcoma cells, C33A and HeLa cervical cancers, and Saos-2 osteo-

C and **D:** The CDK4 inhibitor AG12275 induced growth arrest in HCT 116 colon carcinoma cells.

C: AG12275 selectively inhibits CDK4 kinase activity. CDK4-associated Rb kinase activity and CDK2-associated histone H1 kinase activity were measured in extracts from HCT 116 cells (Ponasterone A minus). Synchronized cells were treated with either DMSO (0) or 0.6 μM AG12275, respectively. Samples were coprecipitated with either CDK4 or two antibodies.

D: Flow cytometric cell cycle analysis treated with indicated concentration of AG12275 and Ponasterone A. Percentage of G0/G1 population was shown. Little enhancement was seen by p27 Kip1 expression.

E–H: p27 Kip1 is not required for the U0126-induced growth arrest.

E: Western blot analysis using lysates from SW480 colon carcinoma cells. Growing cells were treated with indicated concentration of either mismatch (MM) oligonucleotide or p27 kip1 antisense (AS) oligonucleotide and cultured in the medium for 24 hr.

F–H: Synchronized SW480 cells were treated with combination of DMSO or U0126, and MM oligonucleotide (−) or p27 kip1 AS oligonucleotide (+). Cells were cultured in the medium for 24 hr.

F: Histone H1-associated CDK2 kinase assay.

G: Western blot analysis.

H: Cell cycle distribution after p27 Kip1 depletion in SW480 cells.

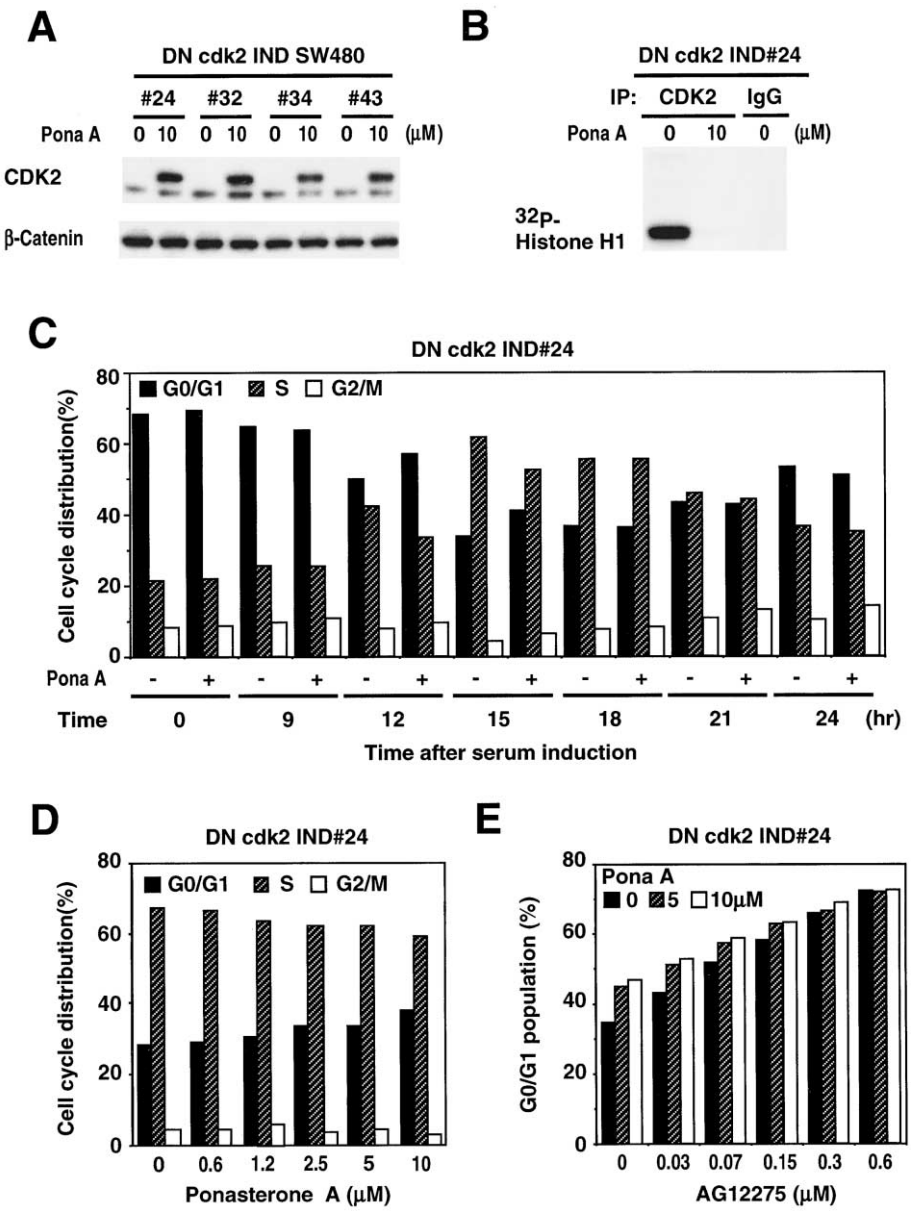

Figure 5. CDK2 activity is dispensable for G1/S transition in colon cancers

A–D: Expression of D145N DN form of CDK2 did not cause growth arrest in colon cancers.
A: DN CDK2 ecdysone-inducible (IND) SW480 cell lines.
B: CDK2-associated histone H1 kinase assay on DN CDK2-overexpressed SW480 cells. Samples were coprecipitated with either CDK2 or control IgG antibody.
C–E: Flow cytometric cell cycle analysis.
C: DN cdk2 IND SW480 line #24 cells were serum starved for 48 hr in the absence (−) or presence (+) of 10 μM Ponasterone A and then stimulated by the addition of 10% FBS containing media with or without 10 μM Ponasterone A to allow synchronous progression. Samples were collected at indicated time points.
D: Cell cycle distribution after DN CDK2 induction in SW480 cells.
E: Combination of AG12275 and DN CDK2 had no effect on the cell cycle profile.

sarcomas. First, we evaluated levels of CDK2 protein and their kinase activities after 48 hr from transfections either with AS oligonucleotides or small interfering (si) RNA. Growing U-2 OS and C33A were transfected either with mismatch (MM) oligonucleotide or cdk2 AS oligonucleotide (Figure 7C). cdk2 AS oligonucleotide but not MM oligonucleotide significantly reduced levels of CDK2 protein. As transfections of MM oligonucleotides showed toxicity in both HeLa and Saos-2 cells, we therefore tested RNA interference on these cells (Figure 7C). We generated four cdk2 siRNA double-stranded oligonucleotides. Among these, #2 cdk2 siRNA significantly reduced expression of CDK2 protein and its kinase activity. Transfection of #5 cdk2 siRNA shows that the endogenous CDK2 expression was not affected; we therefore used #5 siRNA as a control for transfection in the further experiments. To determine effects on cell growth, flow cytometric cell cycle analysis was performed after 48 hr from the transfection with either MM oligonucleotide or cdk2 AS oligonucleotide for U-2 OS and C33A cells, or #5 (control) or #2 siRNA for HeLa and Saos-2 cells (Figure 7D). To our surprise, not only Rb-positive cells (U-2 OS) but Rb-negative cells (C33A, HeLa and Saos-2) failed to growth arrest at G0/G1 following depletion of CDK2 (Heise et al., 1997). This was confirmed by counting viable cell numbers: no difference was observed between MM and AS or control (#5) and siRNA (#2) transfected cultures (data not shown). This suggests that sustained proliferation is possible in the absence of CDK2 protein. Furthermore, U-2 OS cells were treated with the combination of AG12275 with AS cdk2 (Figure 8A). AG12275 induced G1 arrest in a dose-dependent manner; however, little enhancement was seen by depletion of CDK2 expression through AS cdk2, suggesting that partial inhibition of CDK4 did not render U-2 OS cells sensitive to CDK2 inhibition. We suggest that growth inhibition by DN CDK2 reported previously and confirmed above must be due to effects on other components of the cell cycle. For example, DN CDK2 could sequester p21 Cip1 or p27 Kip1 proteins that

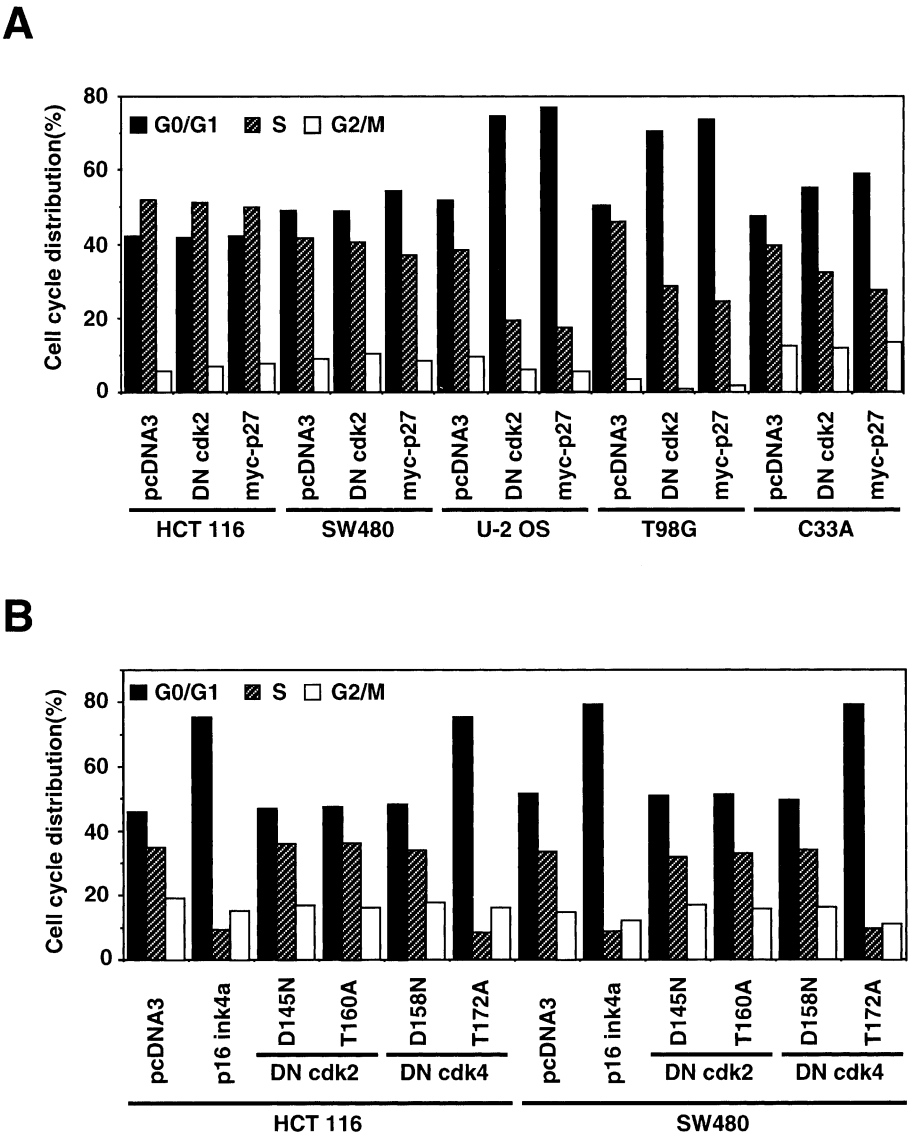

Figure 6. Growth arrest by DN CDKs

A: Growth arrest by a DN CDK2 or Myc-tagged p27Kip1. Cycling HCT 116 and SW480 colon cancers, U-2 OS osteosarcomas, T98G glioblastomas, and C33A cervical cancers were transfected with either 7.5 μg pcDNA3 or either D145N DN cdk2 or Myc-tagged p27 kip1 pcDNA3 expression vector with 2.5 μg pMACS K^k (Miltenvy Biotec).

B: Growth arrest by a DN CDK4. Cycling HCT 116 and SW480 colon cancers were transfected with either 7.5 μg pcDNA3, p16 ink4a, D145N or T160A DN cdk2, or D158N or T172A DN cdk4 pcDNA3 expression vector with 2.5 μg pMACS K^k.

A and **B:** The cells were harvested 24 or 48 hr after transfection and subsequently stained with propidium iodide (PI) containing buffer. Flow cytometric cell cycle analysis was performed with cells expressing the truncated H–$2K^k$.

serve as assembly factors for cyclin D/CDK complexes in these cells. In addition, DN CDK2 has been shown to inhibit CDK1 through association with Cyclin B (Hu et al., 2001).

CDK4 is able to phophorylate Rb even at CDK2 preferred phosphorylation sites

A Recent model showed that phosphorylation of Rb is triggered by CDK4 (6) kinases and probably completed by CDK2 kinases as cells enter S phase (Cheng et al., 1999). As partial inhibition of CDK4 did not render cancer cells sensitive to CDK2 inhibition in HCT 116, SW480, and U-2 OS cells, we tested the possibility that CDK4 activities compensate requirements of CDK2 activities for Rb phosphorylation (Figures 4D, 5E, and 8A). We assessed the phosphorylation status of endogenous Rb protein particularly for CDK2 phosphorylation sites, Ser807/811, where their phosphorylation is thought necessary for G1-S transition (Brugarolas et al., 1999). We treated cells by combining AG12275 with p27 Kip1 induction in HCT 116, DN cdk2 induction in SW480, or AS cdk2 transfection in U-2 OS (Figure 8B). Inhibition of CDK4 but not CDK2 significantly reduced phosphorylation of Rb on Ser807/811 similar to dephosphorylation of Rb as a whole, suggesting that in these Rb-positive cells, increased levels of CDK4 activity induced by MAPK and/or β-catenin may make cdk2 redundant (Tetsu and McCormick, 1999). To test whether other cdks associate with cyclin A or cyclin E following depletion of CDK2, we immunoprecipitated these cyclins and measured associated kinase activity. Using Histone H1 as a substrate, we were unable to detect other associated kinase activities (Figure 8C). In addition, we were able to show that Rb-negative C33A cells were able to proliferate continuously in the absence of either CDK2 or CDK4 activity (Figure 8D).

Discussion

CDK4 activity may make CDK2 redundant in colon cancer cells

In this report, we show that CDK2 activity is dispensable for growth of mammalian cells. CDKs was inhibited or depleted by overexpression of p27 Kip1, expression of DN CDK2, antisense

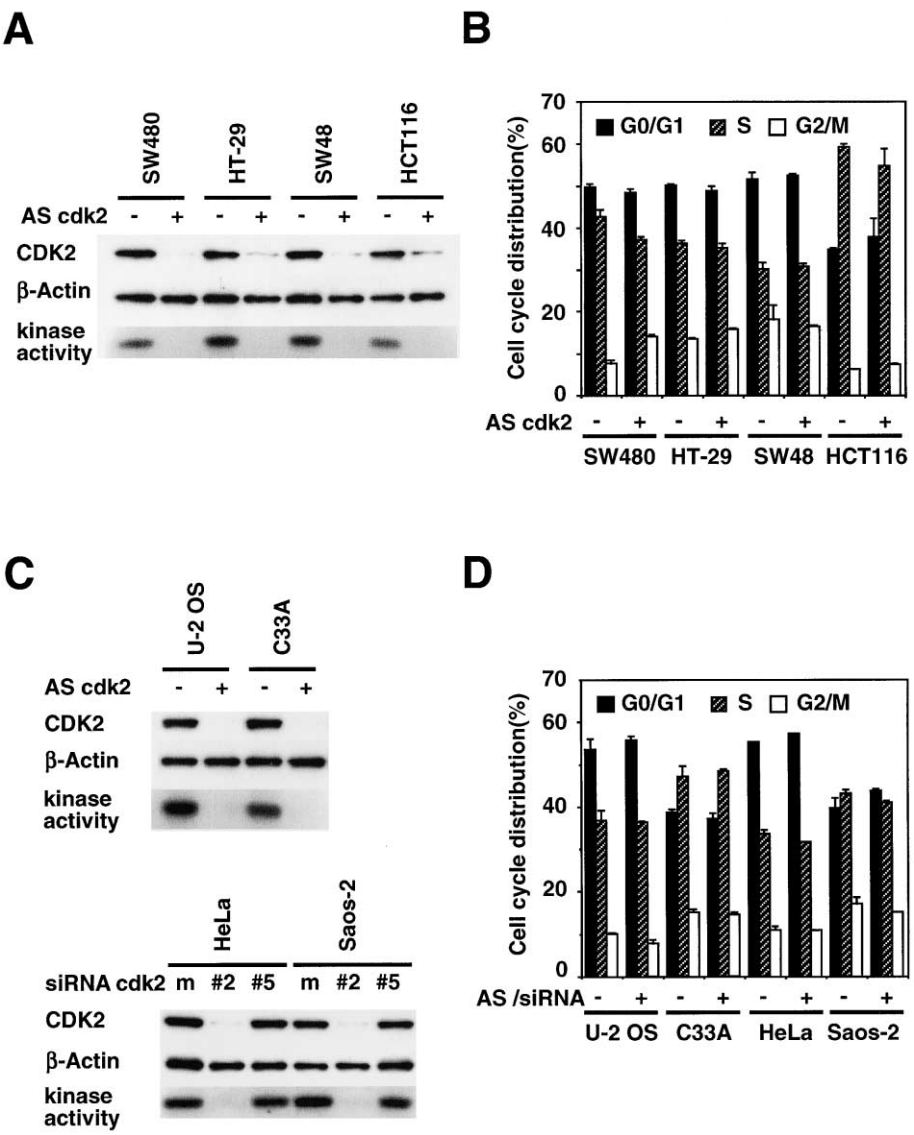

Figure 7. Proliferation of cancer cells does not depend on CDK2 activity

A and **B:** CDK2 activity is dispensable in colon cancers. Growing SW480, HT-29, SW48, and HCT 116 colon carcinoma cells were treated with 300 nM of either mismatch oligonucleotide (−) or cdk2 antisense (+) oligonucleotide and cultured in the medium for 48 hr.

A: Western blots analysis (CDK2 and β-actin) and CDK2-associated histone H1 kinase assay (kinase activity).

B: Flow cytometric cell cycle analysis.

C and **D:** CDK2 activity is dispensable in osteosarcomas and cervical cancers. Growing U-2 OS osteosarcoma cells and C33A cervical cancer cells were treated with 300 nM of either mismatch oligonucleotide (−) or cdk2 antisense (+) oligonucleotide and cultured in the medium for 48 hr. Growing HeLa cervical cancer cells and Saos-2 osteosarcoma cells were treated with mock (m) or 120 nM of #2 (+) or #5 (−) cdk2 small interfering (si) RNA doublestrand oligonucleotides and cultured in the medium for 48 hr.

C: Western blots analysis (CDK2 and β-actin) and CDK2-associated histone H1 kinase assay (kinase activity).

D: Flow cytometric cell cycle analysis.

oligos against cdk2, or by siRNA. Previous reports implicating an essential role for CDK2 in cancer cells relied on effects of DN constructs and expression of p27 Kip1: here we suggest that these proteins have additional effects that are not related directly to CDK2 inhibition. Substrates for CDK2 include Rb and proteins involved in centrosome duplication. However, it has not been shown that phosphorylation of these substrates by CDK2 is essential for cell cycle progression. Here we show that phosphorylation of CDK2-preferred recognition sites Ser807/811 on Rb occurs in the absence of CDK2. These cancer cells have high levels of CDK4 activity, through loss of p16 INK4a, upregulation of cyclin D1 (through Ras and β-catenin signaling), and expression of the assembly factor p21 Cip1: perhaps for these reasons, CDK4 is able to phosphorylate Rb on CDK2-preferred sites (Figure 8B).

We also suggest that the requirement of CDK2 activity for centrosome duplication is reduced in colon cancer cells. Recently, it has been shown that CDK2 is required for duplication of the centrosome and spindle pole body, particularly for phosphorylation of nucleophosmin NO38/B23 and Mps1 protein kinase, which were identified as centrosomal substrates for CDK2 (Okuda et al., 2000; Stucke et al., 2002). Interestingly, centrosome amplification in tumors has been linked to genetic changes in the p53 pathway, including mutations in p53 itself, its regulators MDM2, and downstream targets p21 Cip1 and Gadd45 (Nigg, 2001; Tarapore et al., 2001). All colon cancer cells and U-2 OS cells we studied here have mutations in either p53 or the MDM2 regulator p14ARF (Heise et al., 1997; Burri et al., 2001). Centrosome amplification was also observed in response to overexpression of aurora-A kinase (Zhou et al., 1998). Aurora-A kinase is overexpressed in cell lines and primary colon cancers including SW480 cells (Bischoff et al., 1998). Taken together, we suggest that CDK2 activity is dispensable for both DNA replication and centrosome duplication in these Rb-positive colon cancer cells. Antisense (AS) cdk2 and siRNA experiments in this report suggest that CDK2 activity may also be unnecessary in Rb-negative cells, although we believe further studies are required to resolve this issue. We conclude that CDK2 activity is dispensable in cancer cells and that selective CDK2 inhibition does not offer a promising strategy for cancer therapeutics.

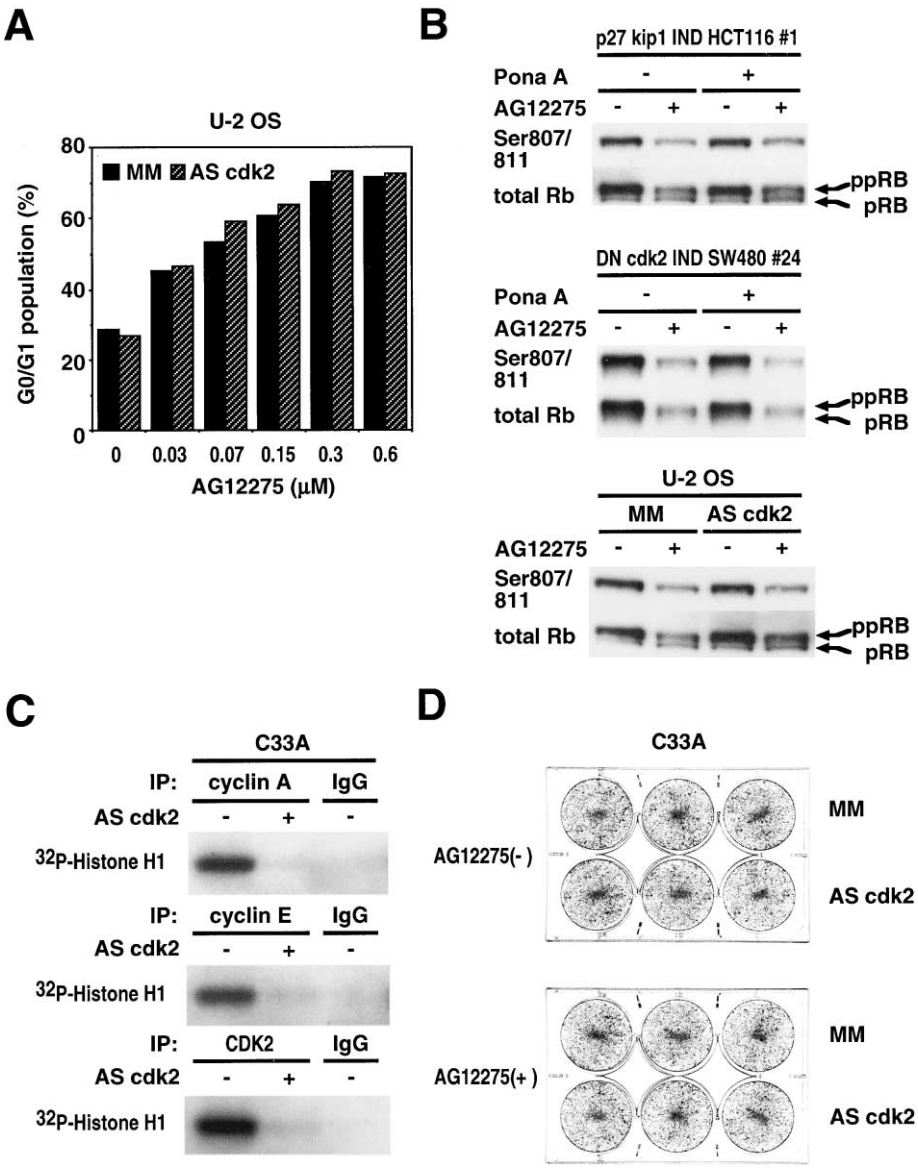

Figure 8. Proliferation of cancer cells despite CDK2 inhibition

A: Combination of AG12275 and AS cdk2 in U-2 OS cells had no effects on the cell cycle profile.
B: CDK4 phophorylates Rb at CDK2 preferential phosphorylation sites Ser807/811. Phosphorylation status for the endogenous Rb expression was analyzed with total Rb antibody and Rb phosphorylation antibodies specific to CDK2. pRb and ppRb stands for nonphosphorylated and phosphorylated forms of Rb, respectively. Cells were treated in the combination of AG12275 with p27 Kip1 induction in HCT 116, DN cdk2 induction in SW480, or AS cdk2 transfection in U-2 OS. Ten micromolar of Ponasterone A and 0.6 μM of AG12275 were used.
C and D: C33A cells proliferate despite CDK2, CDK4, and CDK6 inhibition.
C: cyclin A, cyclin E, and CDK2 associated histone H1 kinase assays on AS cdk2-treated C33A cells. C33A cells were treated with either MM (−) or AS cdk2 (+). Samples were coprecipitated either with cyclin A, cyclin E, CDK2, or control IgG antibodies.
D: Colony formation assay in the combination of AG12275 and AS cdk2 in C33A cells. One hundred single cells from C33A were cultured in the absence (−) or presence (+) of AG12275 and MM or AS cdk2. Cultured after 21 days, cells were stained with 0.5% crystal violet containing 20% ethanol. Triplicate cell cultures are shown.

Experimental procedures

Chemicals, site-directed mutagenesis, cell culture, and establishment of inducible cell lines

The MEK inhibitors, U0126 (Promega) and PD 184352 (Calbiochem and Upstate), the CDK4 inhibitor AG12275, and the ecdysone analog Ponasterone A (Invitrogen) were suspended in DMSO. CDK T loop mutant forms, T160A cdk2 and T172A cdk4, were generated using QuickChange site-directed mutagenesis kit according to the instructions (Stratagene). SW480, HT-29, SW 48, DLD-1, HCT 116, U-2 OS, T98G, Saos-2, HeLa, and C33A were obtained from the American Type Culture Collection. Ecdysone-inducible cell lines were established using the Ecdysone-Inducible Mammalian Expression System (Invitrogen). pIND-inducible expression vector resistant to Hygromycine which contains either myc epitope-tagged p27 kip1 and HA epitope-tagged D145N DN cdk2 was transfected by using FuGENE6 (Roche) upon HCT 116 or SW480 cell lines carrying ecdysone response receptor, respectively (Wakita et al., 2001). Ten or fifty single-cell derived independent drug-resistant colonies were cloned and screened for each gene. Exogenous expression levels of each of these genes were monitored by Western blotting. The highest induced gene expression cell line was chosen for further study. Chimeric phosphorothioate antisense oligonucleotides directed against p27 kip1 (CTCCGCTAACCCCGTCTGGC) and 8 bp mismatch oligonucleotides (CGCCTCGACCCACTTCGGTC), and cdk2 (TGC GATAACAAGCTCCGTCC) and 8 bp mismatch oligonuleotides (GGCTAC AATATGCACTGCCC), were synthesized as described previously (Gottschalk et al., 2001; ISIS Pharmaceuticals). Antisense and mismatch oligonucleotides were transfected by using Lifofectin (Invitrogen). cdk2 small interfering (si) RNA oligonucleotide target sites were selected and generated as recommended (Xeragon, www.xeragon.com). siRNAs were transfected by using Oligofectamine (Gibco, Invitrogen).

Western blot analysis

Protein was prepared with cell lysis buffer 50 mM Tris HCl (pH 8.0), 120 mM NaCl, 0.25%NP-40, and 0.1% SDS containing the protease inhibitor cocktail, Complete Mini (Roche) to obtain whole-cell lysates. Twenty micrograms of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Eight (Rb blotting) or Twelve percent (others) acrylamide gel was used. Western blots were developed by enhanced chemiluminescence (Amersham). Following monoclonal and polyclonal primary and secondary antibodies, horseradish peroxidase (HRP) conjugated antibodies were used. Active (phosphorylated)-ERK (Promega, E-4, Santa Cruz), Pan (p42 and 44)-ERK (Promega), cyclin A (C-19, Santa Cruz), cyclin

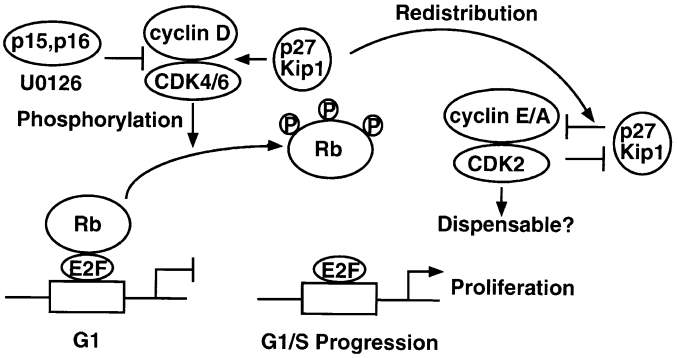

Figure 9. The mechanism of G1/S transition

Rb protein is completely phophorylated and inactivated by CDK4 (6) kinases late in G1. Phosphorylation of the Rb protein releases E2F activity and E2F in turn activates transcription of a number of genes involved in cell proliferation. Ink4 proteins including p15 Ink4b and p16 Ink4a inhibit CDK4 (6) kinases. p21 Cip1/p27 Kip1 family members, which promote the association of cyclin D with CDK4 (6), are redistributed from CDK4 (6) to CDK2 following increases in expression of Ink4 protein or inhibition of the Ras/Raf/MEK/MAP kinase pathway, e.g., U0126. Increased levels of CDK2 activity could accelerate degradation of p27 Kip1 (Vlach et al., 1997); however, we question the role of CDK2 in cell proliferation.

D1 (A-12 and H-295, Santa Cruz), cyclin D2 (C-17, Santa Cruz, Ab-1, Calbiochem), cyclin D3 (Transduction), cyclin E (Ab-1, Calbiochem), CDK1/CDC2 (Transduction), CDK2 (D-12, Santa Cruz), CDK4 (H-303, Santa Cruz, Ab-2, Calbiochem, Transduction), CDK6 (B-10, Santa Cruz), p16 Ink4a (F-12, Santa Cruz), p21 Cip1 (Transduction), p27 Kip1 (Transduction), p57 Kip2 (C-20, Santa Cruz, Ab-2, Calbiochem), total Rb and phosphorylated Rb (Cell Signaling), β-catenin (Transduction), β-actin (Sigma), sheep anti-mouse IgG HRP, sheep anti-rat IgG HRP, and donkey anti-rabbit IgG HRP (Amersham, Roche).

Immunoprecipitation and immunoblotting

Protein was prepared with nonradioactive cell lysis buffer (Cell Signaling) and protease inhibitor cocktail tablet (Roche). Extracts containing 300 μg protein were immunoprecipitated with 2 μg rabbit antibodies to CDK2, 4, 6, p27 Kip1, cyclin A, and cyclin E (M2, H-22, C-21, C-19, C-19/H-432, M-20, Santa Cruz), respectively, at 4°C for 2 hr and collected with 30 μl Protein G PLUS-Agarose (Santa Cruz) at 4°C for 1 hr. After four washes in nonradioactive kinase buffer (Cell Signaling), immune complexes were resuspended with 25 μl sample loading buffer and subjected to SDS-PAGE. Immunoblotting was performed using mouse monoclonal antibodies that we described in the above.

Protein kinase assays

Precipitated immune complex was used for protein kinase assays. Reactions were performed with 25 μl kinase buffer, 50 mM Tris-HCl (pH 8.0), 10 mM β-glycerophosphate, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM $MgCl_2$ containing 20 μM ATP, and 0.37 M Bq γ-^{32}P ATP plus 2 μg histone H1 (Upstate) or recombinant GST-Rb (Santa Cruz) at 30°C for 30 min. Reactions were stopped by adding sample loading buffer and subjected to SDS-PAGE. The gel was dried and subjected to autoradiography.

Cell cycle analysis

Cells were pelleted and resuspended in 1 ml of 0.1% sodium citrate containing 0.3% NP-40, 0.0002 mg/ml RNase, and 50 mg/ml propidium iodide, and incubated for 30 min on ice. The profile of cells in the G0/G1, S, and G2/M phases of the cell cycle were analyzed by the UCSF Cancer Center Cytometry Core, on a FACSCaliber with Cellquest (Becton Dickinson) or ModFit (Verity Software House) Software.

Acknowledgments

The authors gratefully acknowledge M. McMahon, K. Akahane, A. Fattaey, K. Wakita, M. Aonuma, C. Brandts, Y. Hitoshi, and J. Phuchareon for their valuable suggestions and critical discussion. We thank members of McCormick and Stokoe Labs for their helpful support, and the UCSF Comprehensive Cancer Center Genome Analysis and Cytometry Core facilities. We are also grateful to L. Johnson, Onyx Pharmaceuticals, and M. Van den Heuvel and E. Harlow for providing p27 Kip1 and CDK cDNAs, respectively, and N. Dean, ISIS Pharmaceuticals for p27 kip1 and cdk2 antisense and mismatch oligonucleotides. This work was supported by a grant from Daiichi Pharmaceuticals, Tokyo, Japan for the Daiichi Cancer Research Program at UCSF. We dedicate this paper to Frank's father David McCormick, who passed away on Feb 13, 2003.

Received: August 30, 2002
Revised: February 27, 2003

References

Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell *102*, 55–66.

Bischoff, J.R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., et al. (1998). A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. *17*, 3052–3065.

Blain, S.W., Montalvo, E., and Massague, J. (1997). Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. J. Biol. Chem. *272*, 25863–25872.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J. *18*, 5321–5333.

Brugarolas, J., Moberg, K., Boyd, S.D., Taya, Y., Jacks, T., and Lees, J.A. (1999). Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. Proc. Natl. Acad. Sci. USA *96*, 1002–1007.

Buolamwini, J.K. (2000). Cell cycle molecular targets in novel anticancer drug discovery. Curr. Pharm. Des. *6*, 379–392.

Burri, N., Shaw, P., Bouzourene, H., Sordat, I., Sordat, B., Gillet, M., and Schorderet, D., Bosman, F.T., and Chaubert, P. (2001). Methylation silencing and mutations of the p14ARF and p16INK4a genes in colon cancer. Lab. Invest. *81*, 217–229.

Cheng, M., Olivier, P., Diehl, J.A., Fero, M., Roussel, M.F., Roberts, J.M., and Sherr, C.J. (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. *18*, 1571–1583.

Coleman, K.G., Sedman, S.A., Brinkley, P., Price, S., and Webster, K.R. (1997). Identification of CDK4 sequences involved in cyclin D1 and p16 binding. J. Biol. Chem. *272*, 18869–18874.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105.

Dirks, P.B., Patel, K., Hubbard, S.L., Ackerley, C., Hamel, P.A., and Rutka, J.T. (1997). Retinoic acid and the cyclin dependent kinase inhibitors synergistically alter proliferation and morphology of U343 astrocytoma cells. Oncogene *15*, 2037–2048.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. *273*, 18623–18632.

Gottschalk, A.R., Basila, D., Wong, M., Dean, N.M., Brandts, C.H., Stokoe, D., and Haas-Kogan, D.A. (2001). p27Kip1 is required for PTEN-induced G1 growth arrest. Cancer Res. *61*, 2105–2111.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D.D., and Kirn, D.H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nat. Med. *3*, 639–645.

Herrera, R.E., Sah, V.P., Williams, B.O., Makela, T.P., Weinberg, R.A., and Jacks, T. (1996). Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. Mol. Cell. Biol. *16*, 2402–2407.

Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993–1006.

Hu, B., Mitra, J., van den Heuvel, S., and Enders, G. (2001). S and G2 phase roles for Cdk2 revealed by inducible expression of a dominant-negative mutant in human cells. Mol. Cell. Biol. *21*, 2755–2766.

Jiang, H., Chou, H.S., and Zhu, L. (1998). Requirement of cyclin E-Cdk2 inhibition in p16(INK4a)-mediated growth suppression. Mol. Cell. Biol. *18*, 5284–5290.

Katayose, Y., Kim, M., Rakkar, A.N., Li, Z., Cowan, K.H., and Seth, P. (1997). Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res. *57*, 5441–5445.

Kato, J.Y., Matsuoka, M., Strom, D.K., and Sherr, C.J. (1994a). Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. Mol. Cell. Biol. *14*, 2713–2721.

Kato, J.Y., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. (1994b). Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell *79*, 487–496.

Kitagawa, M., Higashi, H., Jung, H.K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. (1996). The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J. *15*, 7060–7069.

Koh, J., Enders, G.H., Dynlacht, B.D., and Harlow, E. (1995). Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature *375*, 506–510.

Kwon, T.K., and Nordin, A.A. (1997). Overexpression of cyclin E and cyclin-dependent kinase inhibitor (p27Kip1): effect on cell cycle regulation in HeLa cells. Biochem. Biophys. Res. Commun. *238*, 534–538.

Lukas, J., Bartkova, J., Rohde, M., Strauss, M., and Bartek, J. (1995a). Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. *15*, 2600–2611.

Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995b). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature *375*, 503–506.

Madema, R.H., Herrera, R.E., Lam, F., and Weinberg, R.A. (1995). Growth suppression by p16 ink4 requires functional retinoblastoma protein. Proc. Natl. Acad.Sci. USA *92*, 6289–6293.

Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: A critical decision in cancer. Nat. Rev. Cancer *1*, 222–231.

Matsuoka, M., Kato, J.Y., Fisher, R.P., Morgan, D.O., and Sherr, C.J. (1994). Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. Mol. Cell. Biol. *14*, 7265–7275.

McConnell, B.B., Gregory, F.J., Stott, F.J., Hara, E., and Peters, G. (1999). Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. Mol. Cell. Biol. *19*, 1981–1989.

Naruse, I., Hishino, H., Dobashi, K., Minato, K., Saito, R., and Mori, M. (2000). Over-expression of p27 Kip1 induces growth arrest and apoptosis mediated by changes of pRB expression in lung cancer cell lines. Int. J. Cancer *88*, 377–383.

Nigg, E.A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. Nat. Rev. Mol. Cell Biol. *2*, 21–32.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol. Cell. Biol. *121*, 2612–2624.

Okuda, M., Horn, H.F., Tarapore, P., Tokuyama, Y., Smulian, A.G., Chan, P.K., Knudsen, E.S., Hofmann, I.A., Snyder, J.D., Bove, K.E., and Fukasawa, K. (2000). Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell *103*, 127–140.

Pagano, M.R., Pepperkok, R., Verde, F., Ansonge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. *11*, 961–971.

Perez-Roger, I., Kim, S.H., Griffiths, B., Sewing, A., and Land, H. (1999). Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J. *18*, 5310–5320.

Polyak, K., Kato, J.Y., Solomon, M.J., Sher, C.J., Massague, J., Robert, J.M., and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. *8*, 9–22.

Reed, S.I. (1997). Control of the G1/S transition. In Checkpoint Controls and Cancer, Cancer Surveys *29*, M. Kastern, ed., (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 7–23.

Reynisdottir, I., and Massague, J. (1997). The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev. *11*, 492–503.

Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., et al. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. *5*, 810–816.

Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103–112.

Sherr, C.J., and Roberts, J.M. (1999).CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501–1512.

Stucke, V.M., Sillje, H.H., Arnaud, L., and Nigg, E.A. (2002). Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. EMBO J. *21*, 1723–1732.

Swarbrick, A., Lee, C.S., Sutherland, R.L., and Musgrove, E.A. (2000). Cooperation of p27(Kip1) and p18(INK4c) in progestin-mediated cell cycle arrest in T-47D breast cancer cells. Mol. Cell. Biol. *20*, 2581–2591.

Tarapore, P., Horn, H.F., Tokuyama, Y., and Fukasawa, K. (2001). Direct regulation of the centrosome duplication cycle by the p53-p21Waf1/Cip1 pathway. Oncogene *20*, 3173–3184.

Tetsu, O., and McCormick, F. (1999). β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422–426.

Tsai, L.-H., Lees, E., Faha, B., Harlow, E., and Riabowol, K. (1993). The cdk kinase is required for the G1-to-S transition in mammalian cells. Oncogene *8*, 1593–1602.

Tsutsui, T., Hesab, B., Moons, D.S., Pandolfi, P.P., Hansel, K.S., Koff, A., and Kiyokawa, H. (1999). Targeted disruption of CDK4 delays cell cycle entry with enhanced p27 (Kip1) activity. Mol. Cell. Biol. *19*, 7011–7019.

Toogood, P.L. (2001). Cyclin-dependent kinase inhibitors for treating cancer. Med. Res. Rev. *6*, 487–498.

Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell *78*, 67–74.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science *262*, 2050–2054.

Vlach, J., Hennecke, S., and Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. *16*, 5334–5344.

Wakita, K., McCormick, F., and Tetsu, O. (2001). Method for screening ecdysone-inducible stable cell lines. Biotechniques *31*, 414–418.

Yang, H.-Y., Shao, R., Hung, M.-C., and Lee, M.-H. (2001). p27 Kip1 inhibits HER2/neu-mediated cell growth and tumorigenesis. Oncogene *20*, 3695–3702.

Zarkowska, T., and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. *272*, 12738–12746.

Zhou, H., Kuang, J., Zhong, L., Kuo, W.L., Gray, J.W., Sahin, A., Brinkley, B.R., and Sen, S. (1998). Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet. *20*, 189–193.