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Induction of p21 by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities

Guangan He[1], Zahid H Siddik[1], Zaifeng Huang[1], Ruoning Wang[1], John Koomen[2], Ryuji Kobayashi[2], Abdul R Khokhar[1] and Jian Kuang*[,1]

[1]*Department of Experimental Therapeutics, The University of Texas, MD Anderson Cancer Center, Box 019, 1515 Holcombe Blvd, Houston, TX 77030, USA;* [2]*Department of Molecular Pathology, The University of Texas, MD Anderson Cancer Center, Box 019, 1515 Holcombe Blvd, Houston, TX 77030, USA*

DNA damage often activates the p53–p21 pathway and causes G_1-phase arrest in mammalian cells. Although there is ample evidence that p21 induction by p53 leads to Cdk2 inhibition, it is unclear whether this checkpoint event also leads to Cdk4 inhibition. Diaminocyclohexane(*trans*-diacetato)(dichloro) platinum(IV) (DAP), a platinum-based coordination complex, is a DNA-damaging agent that is effective against a variety of tumor cells resistant to the parental drug cisplatin. Our previous studies established that treatment of human cancer cells with low effective concentrations of DAP specifically activates the G_1-phase checkpoint and simultaneously inhibit Cdk4 and Cdk2 activities. Here we demonstrate that DAP treatment of human cancer cells activates the p53–p21 pathway without activating other known mechanisms that inhibit Cdk4 and Cdk2 activities. The induced p21 binds to both the Cdk4/cyclin D and Cdk2/cyclin E complexes and inhibits both of their kinase activities. Conversely, inhibition of p21 induction by cycloheximide or by p21 gene deletion prevents DAP-induced inhibition of Cdk4 and Cdk2 activities. Attenuated p53 expression and p21 induction also eliminates DAP-induced G_1-phase arrest and inhibition of Cdk4 and Cdk2 activities. Together, these findings establish that activation of the p53–p21 pathway is responsible for the DAP-induced G_1-phase checkpoint response and provide the first solid evidence that p21 induction by p53 during a DNA damage-induced G_1-phase checkpoint response inhibits both Cdk4 and Cdk2 activities.
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Introduction

Eucaryotic cells respond to DNA damage by halting their cell cycle progression at G_1, S, and/or G_2 phase. This results from activation of cell cycle checkpoints, which are signal transduction pathways that generate signals to inhibit key cell cycle regulators, most notably the cyclin-dependent kinase (Cdk) complexes that govern cell cycle progression (Elledge, 1996; O'Connor, 1997; Bartek and Lukas, 2001a). Activation of different checkpoints involves inhibition of activities of different Cdk complexes: Cdk4/cyclin D and Cdk2/cyclin E for G_1-phase checkpoint, Cdk2/cyclin A for S-phase checkpoint, and Cdc2/cyclin A and Cdc2/cyclin B for G_2-phase checkpoint. DNA lesions caused by different damaging agents may activate different cell cycle checkpoints and inhibit different Cdk activities (Attardi *et al*., 2004; Buscemi *et al*., 2004; Sancar *et al*., 2004). Previous studies have established that DNA damage-induced Cdk inhibition can be caused by downregulation of cyclins (Muschel *et al*., 1991, 1992; Datta *et al*., 1992; Poon *et al*., 1996; Agami and Bernards, 2000; Miyakawa and Matsushime, 2001), inhibition of complex formation between a Cdk and its cyclin partner (Zhan *et al*., 1999), induction of inhibitory tyrosine phosphorylation of Cdks (Terada *et al*., 1995; Jin *et al*., 1996; Poon *et al*., 1996; Blasina *et al*., 1997; Rhind *et al*., 1997; Ye *et al*., 1997), inhibition of activating phosphorylation of Cdks (Smits *et al*., 2000), or induction of the Cdk inhibitor p21 by p53 (Dulic *et al*., 1994; el-Deiry *et al*., 1994).

Cisplatin, a commonly utilized chemotherapeutic agent, is a simple platinum-based coordination complex that forms adducts with DNA and causes DNA strand breaks (Cohen and Lippard, 2001; Siddik, 2003). Previous studies by others and us have established that cisplatin treatment of human cancer cells primarily causes G_2-phase arrest irrespective of whether cells have a functional p53 (Strathdee *et al*., 2001). Abrogation of the G_2-phase checkpoint response enhances cisplatin-induced cytotoxicity (Wang *et al*., 1996; Eastman *et al*., 2002b). 1*R*,2*R*-diaminocyclohexane(*trans*-diacetato) (dichloro) platinum(IV) (DAP) is a more complex platinum-based coordination complex than cisplatin that also forms adducts with DNA and causes DNA strand breaks (Kido *et al*., 1993). Because DAP is effective against multiple types of cisplatin-resistant cancer cells (Kido *et al*., 1993; Siddik *et al*., 1994), it is a prime candidate for evaluation in second-line chemotherapy of

*Correspondence: J Kuang; E-mail: jkuang@mdanderson.org
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cisplatin-relapsed patients. Biochemical pharmacology data of DAP indicated that the antitumor advantages of this agent are not due to more efficient cell entry or increased DNA adduct formation than in the case of cisplatin (Kido *et al*., 1993), implying that DAP-induced DNA lesions are different in structure from those produced by cisplatin and thus trigger different cellular responses. Consistent with this speculation, characterization of cell cycle effects of DAP clearly demonstrated that DAP preferentially inhibits G_1-phase progression in a p53-dependent manner and that elimination of the p53-dependent G_1-phase checkpoint response reduces DAP-induced cytotoxicity (Hagopian *et al*., 1999). The clear difference between DAP and cisplatin in the checkpoint effect and the impact of the checkpoint responses on cell survival raise the possibility that the preferential G_1-phase checkpoint response induced by DAP contributes to its unique antitumor activity.

To understand the unique cell cycle checkpoint response induced by DAP, it is essential to determine how DAP effectively activates the G_1-phase checkpoint. Our previous investigation using ovarian cancer A2780 cells as a model system established that low effective concentrations (0.2–0.6 μM) of DAP specifically inhibited G_1-phase progression and that the G_1-phase arrest was associated with simultaneous inhibition of Cdk4 and Cdk2 activities (Kuang *et al*., 2001). Following these findings, the objective of the present study was to identify the mechanism(s) that is responsible for coordinated inhibition of Cdk4 and Cdk2 activities during the DAP-induced G_1-phase checkpoint arrest.

Previous studies have established that tyrosine phosphorylation of Cdk2 and p21 binding to the Cdk2 complex are the major mechanisms responsible for Cdk2 inhibition following activation of the G_1-phase checkpoint by DNA damage (Bartek and Lukas, 2001a, b). The increased Cdk2 tyrosine phosphorylation results from degradation of cell division control protein 25A (Cdc25A), which follows its phosphorylation by ataxia-telengiectasia mutated protein (ATM)-activated checkpoint kinase 2 (Chk2) in the case of ionizing radiation or by ATR-activated checkpoint kinase 1 (Chk1) in the case of UV irradiation (Mailand *et al*., 2000; Falck *et al*., 2001). Induction of Cdk2 tyrosine phosphorylation causes an acute but transient inhibition of Cdk2 activity in the absence of *de novo* protein synthesis. The increased p21 binding to the Cdk2 complex results from transcriptional activation of p21 by increased p53 activity. This pathway involves *de novo* RNA and protein syntheses and causes delayed but sustained inhibition of Cdk2 activity. Since DAP-induced G_1-phase arrest requires p53 activity, it is likely that DAP-induced inhibition of Cdk2 activity involves binding of p21 to the Cdk2 complex. In contrast to our solid understanding of Cdk2 inhibition, common mechanisms that are responsible for Cdk4 inhibition during DNA damage-induced G_1-phase checkpoint responses have not been identified. Although one previous study showed that mutation of Cdk4 tyrosine phosphorylation site causes defects in UV-induced G_1-phase checkpoint arrest (Terada *et al*., 1995), this finding was not followed up, and it is unclear whether tyrosine phosphorylation of Cdk4 is a common mechanism for Cdk4 inhibition. Other studies showed that ionizing radiation or UV irradiation causes cyclin D degradation and inhibition of Cdk4 activity (Poon *et al*., 1996; Agami and Bernards, 2000). Nevertheless, whereas cyclin D degradation is induced only at high levels of UV irradiation, Cdk4 activity is inhibited in response to both high and moderate levels of UV irradiation (Poon *et al*., 1996). Previous studies also demonstrated that high levels of p21 inhibit both Cdk4 and Cdk2 activities in cell-free systems (Harper *et al*., 1993, 1995; Xiong *et al*., 1993). However, there was no solid evidence that p21 induced in DNA-damaged cells inhibits both Cdk4 and Cdk2 activities, and there was indirect evidence that p21 induction does not inhibit Cdk4 activity (Sherr and Roberts, 1999). Finally, although the Cdk4 binding protein p16 may disrupt the formation of the Cdk4/cyclin D complex and inhibit Cdk4 activity (Serrano *et al*., 1993), p16 levels do not increase during DNA damage-induced G_1-phase checkpoint responses (Shapiro *et al*., 1998, 2000). Thus, to identify the mechanism(s) responsible for coordinated inhibition of Cdk4 and Cdk2 activities during the DAP-induced G_1-phase checkpoint response, it is essential to examine various possible mechanisms that inhibit Cdk activities in the inhibition process.

Results

DAP treatment causes a coordinated inhibition of Cdk4 and Cdk2 activities

To investigate which of the possible mechanisms is responsible for or crucially involved in the DAP-induced inhibition of Cdk4 and Cdk2 activities, our initial approach was to perform a time course treatment of A2780 cells with DAP and identify the molecular changes in the Cdk4 and Cdk2 complexes that coincide with inhibition of their activities in the time course. For this objective, we routinely treated A2780 cells grown to less than 10% of the saturation density with 0.6 μM DAP for 36 h and collected the cells at 6 h intervals. The cells were then extracted for total proteins, which would be utilized for analysing different parameters of the Cdk4 and Cdk2 complexes. As observed in our previous studies (Kuang *et al*., 2001), such treatment of A2780 cells effectively inhibited G_1- to S-phase progression at 18–36 h (Figure 1a), and the inhibition coincided with dramatic and synchronous decrease in Cdk4 and Cdk2 activities (Figure 1b). We should note that (i) use of the cells at low densities was to ensure that experiments could be completed before cell–cell contact might inhibit cell proliferation; (ii) 0.6 μM was a high end concentration of DAP that specifically activates the G_1-phase checkpoint in A2780 cells; and (iii) apoptotic induction was negligible during the first 36 h of DAP treatment (Kuang *et al*., 2001). Thus, molecular changes in the Cdk4 and Cdk2 complexes should not be attributed to cell death or activation of other cell cycle checkpoints.

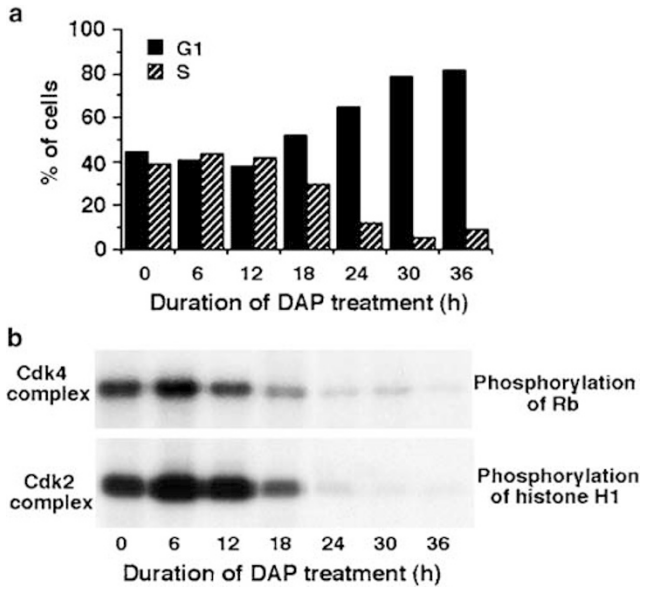

Figure 1 DAP treatment inhibits both Cdk4 and Cdk2 activities. A2780 cells were treated with 0.6 μM DAP for up to 36 h and collected at 6-h intervals. (**a**) The percentage of cells in G_1 and S phases was determined by FACS. (**b**) After the Cdk4 and Cdk2 complexes were immunoprecipitated from crude cell lysates with anti-Cdk4 or anti-Cdk2 antibodies, their kinase activities were measured by phosphorylation of their respective *in vitro* substrates

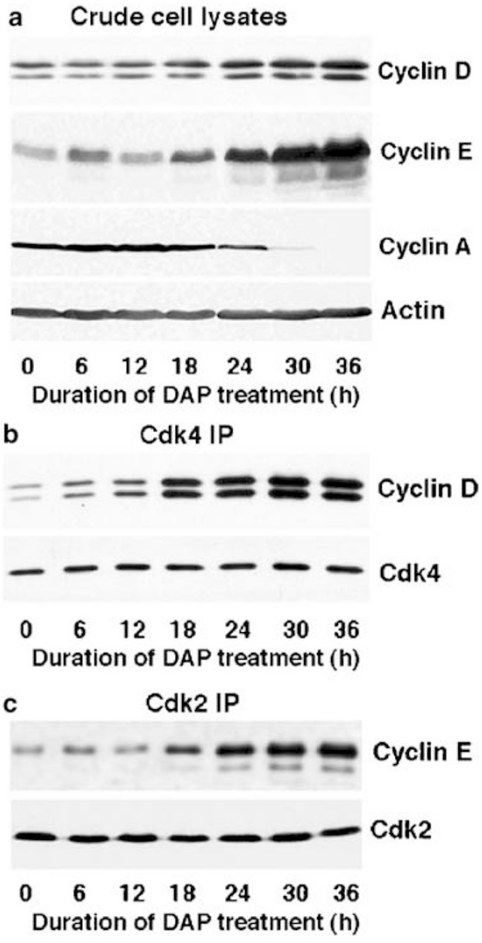

Figure 2 DAP treatment increases the levels of G_1-phase cyclins and G_1-phase Cdk complexes. (**a**) Total proteins were extracted from A2780 cells that had been treated with 0.6 μM DAP for indicated length of time and immunoblotted with antibodies that recognize each of the indicated proteins. (**b**) Cdk4 was immunoprecipitated from the crude cell lysates prepared during the 36-h DAP treatment, and the immunocomplexes were immunoblotted with anti-cyclin D or anti-Cdk4 antibodies. (**c**) Cdk2 was immunoprecipitated from the crude cell lysates prepared during the 36-h DAP treatment, and the immunocomplexes were immunoblotted with anti-cyclin E or anti-Cdk2 antibodies

DAP treatment does not induce degradation of G_1-phase cyclins

To determine whether DAP treatment of A2780 cells induces degradation of G_1-phase cyclins, we immunoblotted crude cell lysates prepared during the 36-h DAP treatment with anti-cyclin D1/2, anti-cyclin E, or anti-cyclin A antibodies. It is known that that D-type cyclins primarily form complexes with Cdk4 (Sherr, 1993) and their levels generally do not oscillate in the cell cycle (Dulic *et al*., 1994; Li *et al*., 1994; Gong *et al*., 1995; Poon *et al*., 1996). In contrast, both cyclins E and A associate with Cdk2 primarily (Dulic *et al*., 1992; Koff *et al*., 1992; Pagano *et al*., 1992), and their levels peak at late G_1 and S phases, respectively (Girard *et al*., 1991; Dulic *et al*., 1992; Koff *et al*., 1992; Pagano *et al*., 1992). As shown in Figure 2a, levels of cyclins D and E progressively increased from 18 to 36 h as Cdk4 and Cdk2 activities progressively decreased. In contrast, the cyclin A level decreased from 18 to 36 h, which correlated with the decrease in the percentages of S-phase cells during this period. Expression of actin, a loading control, was constant throughout the time course (Figure 2a). These results eliminated the possibility that DAP-induced inhibition of G_1-phase Cdk activities involves degradation of G_1-phase cyclins.

DAP treatment does not inhibit formation of the G_1-phase Cdk complexes

To determine whether DAP treatment of A2780 cells inhibits formation of the G_1-phase Cdk complexes, we immunoprecipitated Cdk4 or Cdk2 from crude cell lysates made during the 36-h DAP treatment and analysed the level of its partner cyclin in the immunocomplexes. The results from analysis of the Cdk4 immunoprecipitates showed that while the level of Cdk4 did not change, the level of cyclin D associated with Cdk4 progressively increased from 6 to 30 h (Figure 2b). The results from analysis of the Cdk2 immunoprecipitates showed that while similar amounts of Cdk2 protein were detected throughout the time course, the level of cyclin E associated with Cdk2 progressively increased from 18 to 36 h (Figure 2c). These results eliminated the possibility that DAP-induced inhibition of G_1-phase Cdk activities involves inhibition of the formation of the G_1-phase Cdk complexes.

DAP treatment does not induce inhibitory phosphorylation of G_1-phase Cdks

To determine whether DAP treatment of A2780 cells induces the inhibitory tyrosine phosphorylation of Cdk4 or Cdk2, we immunoprecipitated Cdk4 or Cdk2 from crude cell lysates and immunoblotted it with either anti-Cdk antibodies or multiple anti-phosphotyrosine antibodies. Analysis of the Cdk4 immunoprecipitates showed that tyrosine-phosphorylated Cdk4 was undetectable either before or after the DAP treatment (Figure 3a, top panel), indicating that Cdk4 in this cell line was not or barely phosphorylated on the inhibitory tyrosine. To confirm this speculation, we treated the Cdk4 complex immunoprecipitated from control or 24-h DAP-treated cell lysates with a truncated *Xenopus* Cdc25 protein that contains the phosphatase catalytic domain and examined the effect of such treatment on the Cdk4 activity. Treatment of the Cdc2/cyclin B complex was included as a positive control as it is well established that the inhibitory tyrosine phosphorylation is the major mechanism that renders the Cdc2 kinase complex inactive until mitotic induction (Gould and Nurse, 1989; Morla *et al*., 1989). By logic, if the inhibitory tyrosine phosphorylation indeed played little role in Cdk4 regulation in A2780 cells, Cdc25 treatment of the Cdk4 complex should not affect its kinase activity. The results showed that Cdc25 treatment of the Cdk4/cyclin D complex from control- or DAP-treated cells had little effect on its kinase activity, whereas Cdc25 treatment of the cyclin B/Cdc2 complex greatly increased its kinase activity (Figure 3b). Consistent with lack of Cdk4 staining with multiple anti-phosphotyrosine antibodies, these results eliminated the possibility that induction of the inhibitory tyrosine phosphorylation is a cause of DAP-induced Cdk4 inhibition.

Results from analysis of the Cdk2 immunoprecipitates showed that tyrosine-phosphorylated Cdk2 was detectable at all time points. However, the level of Cdk2 tyrosine phosphorylation did not increase during DAP treatment, and it even decreased at 30–36 h (Figure 3a, bottom panel). In contrast, cisplatin treatment of A2780 cells increased the level of tyrosine-phosphorylated Cdk2 at 12–18 h (data not shown), eliminating the possibility that lack of induction of the inhibitory phosphorylation in Cdk2 by DAP is due to genetic defects in the inducing machinery. Although the reason for the decrease in the Cdk2 tyrosine phosphorylation during DAP treatment was presently not understood and could be due to various reasons, these results clearly indicated that induction of the inhibitory tyrosine phosphorylation is not a cause of DAP-induced Cdk2 inhibition.

The lack of induction of the inhibitory tyrosine phosphorylation in Cdk4 and Cdk2 during DAP treatment led us to speculate that Chk1 and Chk2 were not activated in the process. To test this speculation, we compared levels of total and activated Chk1 or Chk2 in control- and DAP-treated cell lysates. Immunoblotting of crude cell lysates with either anti-Chk1 and anti-Chk2 antibodies or anti-activated Chk1 and anti-activated Chk2 antibodies showed that DAP treatment did not cause any increase in the level of activated Chk1 or Chk2 (Figure 3c). These results supported our speculation.

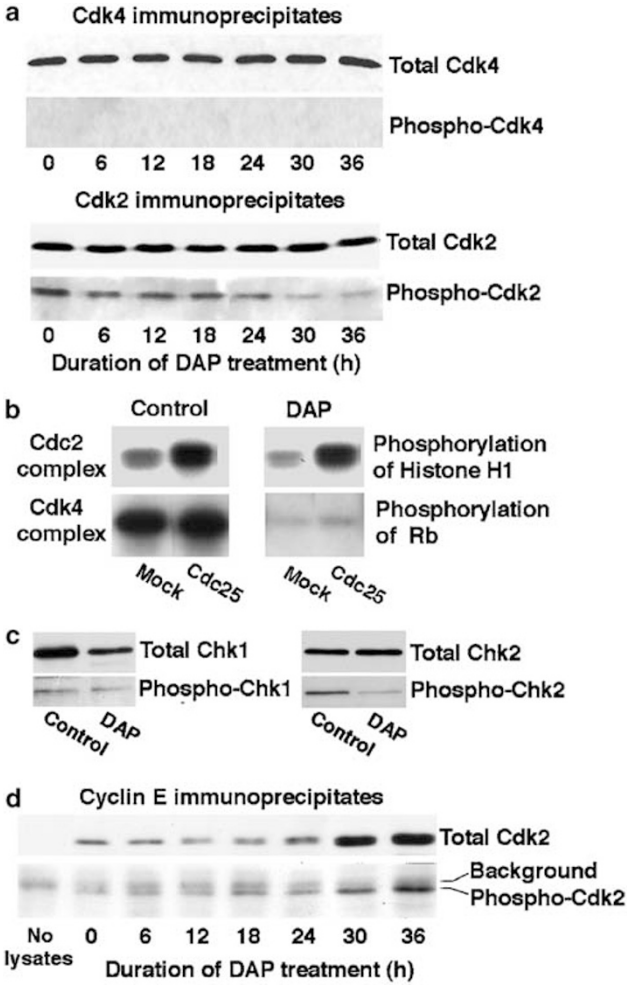

Figure 3 DAP treatment does not induce inhibitory phosphorylation or inhibit activating phosphorylation of G_1-phase Cdks. (**a**) Crude lysates of A2780 cells that had been treated with 0.6 μM DAP for indicated length of time were immunoprecipitated with anti-Cdk4 or anti-Cdk2 antibodies, and the immunocomplexes were immunoblotted with either anti-Cdk antibodies or anti-phosphotyrosine antibody PY99. Note that similar results were obtained with the 4G10 anti-phosphotyrosine antibody. (**b**) After Cdk4 or cyclin B complexes were immunoprecipitated from control- or 24-h DAP-treated A2780 cell lysates, the immunocomplexes were first treated with GST-Cdc25 or control buffer at room temperature for 45 min and then assayed for H1 kinase activity (for the cyclin B complex) or RB kinase activity (for the Cdk4 complex). (**c**) Crude lysates of A2780 cells that had been treated with 0.6 μM DAP for 24 h or mock treated were immunoblotted either with antibodies that recognized the total Chk1 or Chk2 protein or with antibodies that recognized phosphorylated and activated Chk1 or Chk2 protein. (**d**) Crude lysates of A2780 cells that had been treated with 0.6 μM DAP for indicated length of time were immunoprecipitated with anti-cyclin E antibodies, and the immunocomplexes were immunoblotted either with anti-Cdk2 or with anti-phospho-Cdk2 (Thr160) antibodies

DAP treatment does not inhibit activating phosphorylation of G_1-phase Cdks

Previous studies have shown that activating phosphorylation of a Cdk on a conserved threonine residue

(Thr160 in case of human Cdk2) accelerates its migration in SDS–PAGE (Gu *et al*., 1992; Poon and Hunter, 1995). Since no change was observed in the SDS–PAGE mobility of either Cdk4 or Cdk2 irrespective of whether it was examined in crude cell lysates or in the Cdk or cyclin immunoprecipitates, it seems unlikely that DAP treatment inhibits the activating phosphorylation of G_1-phase Cdks. To confirm this, we immunoprecipitated cyclin E from the crude cell lysates prepared during the 36-h DAP treatment and immunoblotted the precipitated proteins with either anti-Cdk2 antibodies or antibodies that specifically recognized Thr160-phosphorylated Cdk2. As shown in Figure 3d, the level of Thr160-phosphorylated Cdk2 correlated with the level of the total Cdk2 protein during the 36-h DAP treatment, confirming that DAP-induced Cdk inhibition does not involve inhibition of activating phosphorylation of G_1-phase Cdks.

DAP treatment activates the p53–p21 pathway

To determine whether DAP treatment of A2780 cells activates the p53–p21 pathway, we measured the level of p53 protein, p21 RNA, and p21 protein during the 36-h DAP treatment by immunoblot analysis of crude cell lysates and Northern hybridization of the total RNA. It was observed that the level of p53 protein significantly increased at 6 h and reached near plateau levels at 12 h (Figure 4a). The level of p21 RNA significantly increased at 12 h and reached near plateau levels at 24 h (Figure 4b). The level of p21 protein slightly increased at 12 h and jumped to near maximal levels at 18 h (Figure 4a). In contrast, the level of the loading control protein actin or the loading control RNA GAPDH did not change. Also, none of the changes was observed in control cells that were cultured in parallel (data not shown). Since the significant inhibition of Cdk4 and Cdk2 activities started at 18 h and maximized at 24 h, these results indicated that DAP treatment activates the p53–p21 pathway prior to inhibiting Cdk4 and Cdk2 activities.

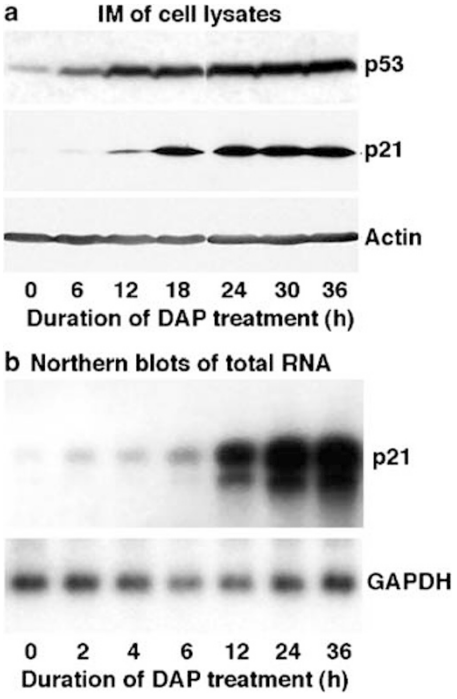

Figure 4 DAP treatment activates p53–p21 pathway. (**a**) Total proteins extracted from A2780 cells that had been treated with 0.6 μM DAP for indicated length of time were immunoblotted with antibodies that recognize each of the indicated proteins. (**b**) The total RNA extracted from A2780 cells that had been treated with 0.6 μM DAP for indicated length of time was hybridized with ^{32}P-labeled p21 and GAPDH cDNAs

DAP-induced p21 binds to both the Cdk4 and Cdk2 complexes efficiently

To determine whether DAP-induced p21 binds to both the Cdk4 and Cdk2 complexes, we immunoprecipitated crude cell lysates prepared during the 36-h DAP treatment with anti-Cdk4 or anti-cyclin D antibodies for the Cdk4 complex and with anti-Cdk2 or anti-cyclin E antibodies for the Cdk2 complex and immunoblotted the precipitated proteins with anti-p21 antibodies. It was observed that the amount of p21 in both the Cdk4 and Cdk2 complexes greatly increased at 18 h and reached plateau levels at 24 h (Figure 5a). In contrast, the level of p27 did not increase in the Cdk4 complex and increased only mildly in the Cdk2 complex (data not shown). These results indicated that DAP-induced p21 binds to both the Cdk4 and Cdk2 complexes.

To determine whether the induced p21 binds to the Cdk4 and Cdk2 complexes in similar quantities, we next measured relative amounts of p21 in the Cdk4 and Cdk2 immunoprecipitates from either control- or DAP-treated cell lysates. p21 was evenly distributed between the Cdk4 and Cdk2 complexes in both cases (Figure 5b), indicating that p21 produced in A2780 cells binds to the Cdk4 and Cdk2 complexes in similar quantities.

To determine whether the induced p21 binds to the Cdk4 and Cdk2 complexes in a quantitative manner, we immunoprecipitated either p27 alone or both p21 and p27 from the 24-h DAP-treated cell lysates and immunoblotted cyclin D, cyclin E, Cdk4, and Cdk2 in both bound and unbound fractions from the immunoprecipitation. Immunodepletion of p27 alone removed $\sim$20% of the Cdk4 and Cdk2 complexes in the cell extracts (data not shown). In contrast, depletion of both p21 and p27 from the DAP-treated cell lysates removed all of the cyclin D and cyclin E and most of Cdk4 and Cdk2 (Figure 5c). The incomplete depletion of Cdk4 and Cdk2 was not surprising since they are usually in excess of their cyclin partners and free Cdks do not bind p21 or p27. These results indicated that DAP-induced p21 quantitatively binds to both the Cdk4 and Cdk2 complexes that do not contain p27.

DAP-induced p21 inhibits both Cdk4 and Cdk2 activities

To investigate the impact of the p21 binding to the Cdk4 and Cdk2 complexes on Cdk4 and Cdk2 activities in DAP-treated cells, we first boiled lysates prepared from 24-h DAP-treated cells to dissociate Cdk inhibitors p21

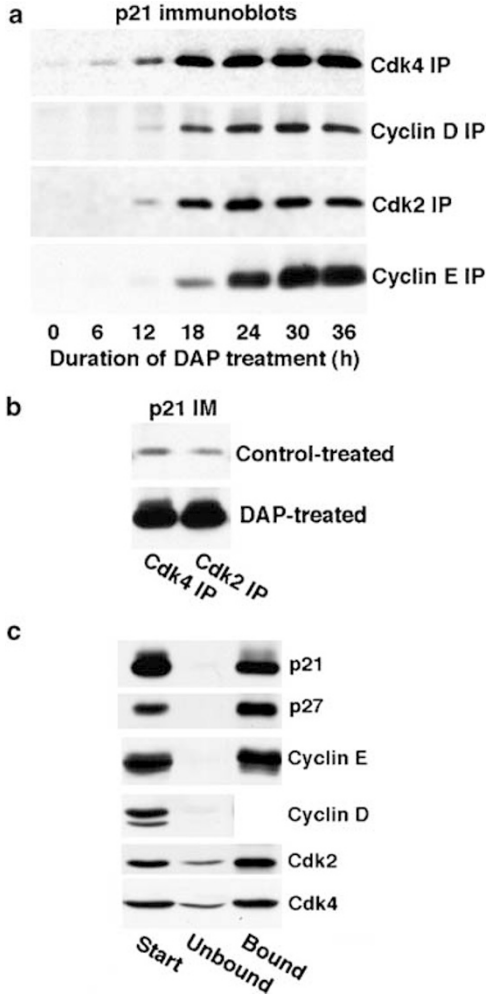

Figure 5 DAP-induced p21 binds to both the Cdk4 and Cdk2 complexes efficiently. (**a**) Cdk4, cyclin D, Cdk2, or cyclin E was immunoprecipitated (IP) from total proteins extracted from A2780 cells that had been treated with 0.6 μM DAP for indicated length of time, and the immunocomplexes were immunoblotted with anti-p21 antibodies. (**b**) Crude lysates of A2780 cells that had been treated with 0.6 μM DAP for 24 h or mock treated were immunoprecipitated with anti-Cdk4 or anti-Cdk2 antibodies, and the immunocomplexes were immunoblotted with anti-p21 antibodies. (**c**) Crude lysates of A2780 cells that had been treated with 0.6 μM DAP for 24 h were immunoprecipitated with a combination of anti-p21 and anti-p27 antibodies. The start crude cell lysates and unbound and bound fractions from the immunoprecipitation were immunoblotted with antibodies that recognized each of the indicated proteins. Note that the bound cyclin D could not be immunoblotted because of interference of the light chain of the precipitating anti-cyclin D antibody

and p27 from the inhibited Cdk4 and Cdk2 complexes. As shown in Figure 6a, such a treatment quantitatively precipitated Cdk and cyclin components of the Cdk4 and Cdk2 complexes but left a significant portion of p21 and p27 in the soluble fraction. We then determined whether the soluble fraction of the heated DAP-treated cell lysates was able to inhibit the activity of the active Cdk4 and Cdk2 complexes immunoprecipitated from control cell lysates. Figure 6b shows that incubation of the Cdk4 and Cdk2 complexes from control cells with the soluble fraction of the boiled DAP-treated cell lysates dramatically inhibited both of their kinase activities. In contrast, unboiled lysates of DAP-treated cells that contain little free Cdk inhibitors did not inhibit Cdk4 or Cdk2 activity. These results indicated that binding of heat-released Cdk inhibitors to the Cdk4 and Cdk2 complexes inhibited their kinase activities. Next, we determined whether the heat-released Cdk inhibitory activity was mainly due to p21. As shown in Figure 6c, if only p27 was immunodepleted from the soluble fraction of the boiled cell lysates, the sample ('Sample-p27' in Figure 6c) still inhibited both Cdk4 and Cdk2 activities. On the other hand, if both p21 and p27 were immunodepleted from the soluble fraction, the sample ('Sample-(p21 + p27)') no longer inhibited Cdk4 or Cdk2 activity. These results indicated that binding of DAP-induced p21 to the Cdk4 and Cdk2 complexes inhibits both of their kinase activities.

p21 induction is required for DAP-induced inhibition of Cdk4 and Cdk2 activities

To determine whether p21 induction is required for DAP-induced Cdk inhibition, we first determined whether protein synthesis is required for DAP-induced inhibition of Cdk4 and Cdk2 activities in A2780 cells. For this, we treated A2780 cells with DAP for 24 h and determined whether inhibition of protein synthesis by 2.0 μM cycloheximide for the last 15 h of the DAP treatment could abrogate DAP-induced inhibition of Cdk4 and Cdk2 activities. As shown in Figure 7a for the Cdk4 complex and Figure 7b for the Cdk2 complex, cycloheximide treatment completely eliminated DAP-induced increase in the level of p21 and the partner cyclin associated with Cdk4 or Cdk2. Along with this effect, DAP treatment no longer inhibited Cdk4 or Cdk2 activity even though the control level of Cdk4 or Cdk2 activity significantly decreased due to reduced levels of cyclin D and E in the absence of new protein synthesis. These results supported the possibility that p21 induction is required for DAP-induced inhibition of both Cdk4 and Cdk2 activities.

To further demonstrate requirement of p21 induction for DAP-induced inhibition of Cdk4 and Cdk2 activities, we utilized an isogenic pair of colon cancer HCT-116 cells that differ only in the p21 gene status (Waldman *et al*., 1995) and compared their cell cycle checkpoint responses to DAP treatment. For this, we first treated this pair of cell lines with different concentrations of DAP for 24 h and examined the end point cell cycle distribution by fluorescence-activated cell sorting (FACS). While treatment of p21-containing HCT-116 cells with 0.5–2 μM DAP increased the percentage of G_1-phase cells and decreased the percentage of S-phase cells in a dose-dependent manner (Figure 8a), treatment of p21-null HCT-116 cells with the same concentrations of DAP did not produce these effects (Figure 8b). We then treated this pair of cell lines with 2 μM DAP for 24 h and monitored changes in the cell cycle distribution at 6-h intervals. Although p21-containing HCT-116 cells showed a progressive

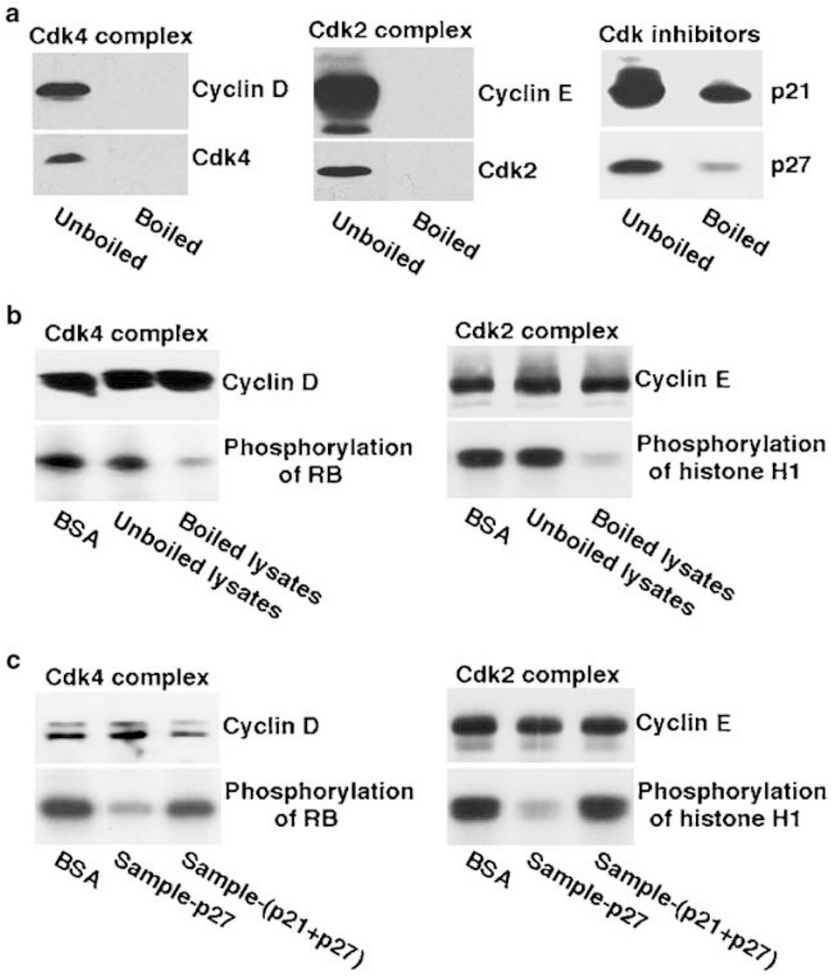

Figure 6 DAP-induced p21 inhibits both Cdk4 and Cdk2 activities. (**a**) Unboiled lysates of 24-h DAP-treated A2780 cells and the soluble and insoluble fractions of the boiled cell lysates were immunoblotted with each of the indicated antibodies. (**b**) Crude lysates of control A2780 cells were immunoprecipitated with anti-Cdk4 or anti-Cdk2 antibodies, and the immunocomplexes were incubated at room temperature for 45 min with BSA, the original DAP-treated cell lysate (unboiled lysates), or the soluble fraction of the boiled DAP-treated cell lysate (boiled lysates). After the immunocomplexes were washed, they were immunoblotted with anti-cyclin D antibodies (for the Cdk4 complex) or anti-cyclin E antibodies (for the Cdk2 complex) and assayed for kinase activity. (**c**) The Cdk4 or Cdk2 immunocomplex from crude lysates of control A2780 cells was incubated at room temperature for 45 min with BSA or the soluble fraction of the boiled DAP-treated cell lysate that was depleted of either p27 alone (sample-p27) or both p21 and p27 (sample-(p21 + p27)). After the immunocomplexes were washed, they were immunoblotted with anti-cyclin D antibodies (for the Cdk4 complex) or anti-cyclin E antibodies (for the Cdk2 complex) and assayed for the kinase activity

accumulation of G_1-phase cells and a continuous decrease in the abundance of S-phase cells in this time course examination (Figure 8c), p21-null HCT-116 cells showed little change in the cell cycle distribution (Figure 8d). Together, these results demonstrated that p21 is required for DAP-induced inhibition of G_1-phase progression. Finally, we treated this pair of HCT-116 cell lines with 2 μM DAP for 24 h and analysed the Cdk4 and Cdk2 complexes from DAP- and control-treated cells. The DAP treatment of p21-containing HCT-116 cells increased the level of p21 and decreased the kinase activity of both the Cdk4 and Cdk2 complexes (Figure 8e). In contrast, the DAP treatment of p21-deleted HCT-116 cells did not affect Cdk4 or Cdk2 activity (Figure 8f). These results confirmed that p21 induction is required for DAP-induced inhibition of both Cdk4 and Cdk2 activities.

Amount of p21 induction determines DAP-induced inhibition of G_1-phase Cdk activities

To determine the generality in the ability of DAP treatment to activate the p53–p21 pathway and inhibit both Cdk4 and Cdk2 activities, we characterized effects of DAP treatment on four additional human cancer cell lines that contain a functional p53, that is, breast cancer MCF-7 cells, fibrosarcoma HT-1080 cells, ovarian cancer OVCA-429 cells, and ovarian cancer 2008 cells. For this, we first treated these cell lines with different concentrations of DAP for 24 h and determined the cell cycle distribution by FACS. Treatment of MCF-7 cells with $\geqslant 0.25\,\mu$M, of OVCA-429 cells with $\geqslant 0.5\,\mu$M, and of HT-1080 cells with $\geqslant 2\,\mu$M DAP increased the percentage of G_1-phase cells and decreased the percentage of S-phase cells (Figure 9a–c), indicating

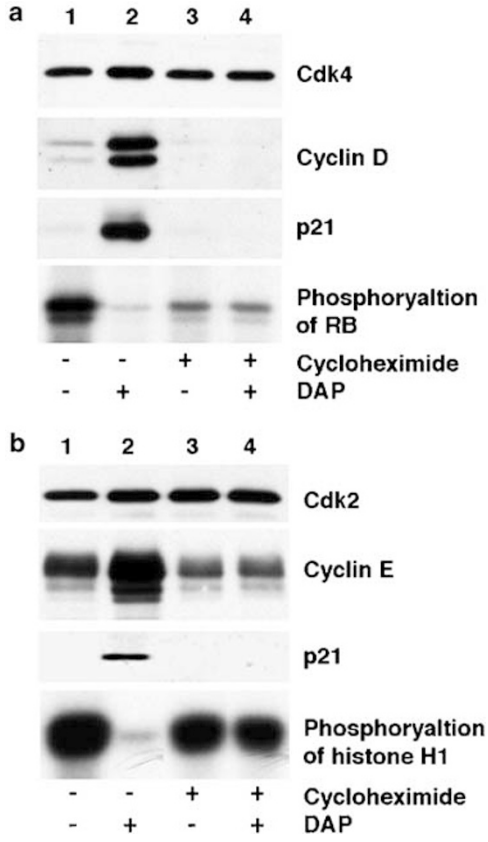

Figure 7 Protein synthesis is required for DAP-induced inhibition of Cdk4 and Cdk2 activities. A2780 cells were cultured for 24 h in the presence or absence of 0.6 μM DAP. For each condition, the last 15-h culture was carried out in the presence or absence of 2.0 μM cycloheximide added at 9 h. (**a**) After Cdk4 was immunoprecipitated from crude lysates of differently treated cells, the immunocomplexes were immunoblotted for Cdk4, cyclin D, and p21 and assayed for RB kinase activity. (**b**) After Cdk2 was immunoprecipitated from crude lysates of differently treated cells, the immunocomplexes were immunoblotted for Cdk2, cyclin E, and p21 and assayed for histone H1 kinase activity

inhibition of G_1- to S-phase progression by DAP in these three cell lines. In contrast, treatment of 2008 cells with $\geqslant$0.25 μM DAP decreased the percentage of G_1-phase cells and increased the percentage of S- and G_2/M-phase cells (Figure 9d), indicating inhibition of S- and G_2/M-phase progressions by DAP in this particular cell line. To confirm lack of G_1-phase effect of DAP treatment on 2008 cells, we first cultured 2008 cells in the presence or absence of 0.5 μM DAP for 24 h and then arrested cell cycle progression at mitosis by nocodazole for 10 h. Determination of the cell cycle distribution by FACS at 0, 5, and 10 h after the nocodazole addition showed that whereas DAP treatment halted progression of S-phase cells into G_2/M phase, it did not retard progression of G_1-phase cells into S phase (Figure 9e). These results confirmed that DAP treatment only inhibits S- and G_2/M-phase progression in 2008 cells.

To determine whether the DAP-induced G_1-phase checkpoint response always correlates with inhibition of both Cdk4 and Cdk2 activities by p21, we treated MCF-7, OVCA-429, HT-1080, and 2008 cells for 24 h with 0.5, 2, 4, and 0.5 μM DAP, respectively, and made crude lysates from control- and DAP-treated cells. We then immunoprecipitated Cdk4 or Cdk2 from each pair of crude cell lysates and measured levels of p21 protein and kinase activity of the immunoprecipitates. DAP treatment of MCF-7, OVCA-429, and HT-1080 cells caused a great increase in the level of p21 and a great decrease in the kinase activity of both the Cdk4 and Cdk2 complexes (Figure 10a–c). In contrast, DAP treatment of 2008 cells produced none of these effects (Figure 10d). These results indicated that inhibition of both Cdk4 and Cdk2 activities by p21 is a general phenomenon associated with DAP-induced G_1-phase arrest.

To understand lack of DAP-induced inhibition of G_1-phase Cdk activities in 2008 cells, we measured relative levels of p53 and p21 proteins in crude lysates of control- and DAP-treated MCF-7, HT-1080, OVCA-429, and 2008 cells. Although DAP treatment increased the level of p53 and p21 in all these four cell lines, both basal and induced levels of p53 and p21 in 2008 cells were much lower than those in the other three cell lines. In contrast, the level of the loading control protein actin did not change (Figure 11a). These results indicated that lack of G_1-phase arrest and inhibition of G_1-phase Cdk activities in DAP-treated 2008 cells are not due to lack of activation of the p53–p21 pathway but due to insufficient amount of p21 induction. To confirm this, we quantitatively immunoprecipitated p21 or both p21 and p27 from DAP-treated 2008 cell lysates and immunoblotted cyclin D and cyclin E in both bound and unbound fractions. Although induced p21 in 2008 cells was able to bind to both cyclin D and cyclin E, its removal, either alone or together with p27, caused little depletion of cyclin D or cyclin E from the DAP-treated cell lysate (Figure 11b). These results confirmed our speculation.

Finally, we determined whether treatment of 2008 cells with higher concentrations of DAP can enhance activation of the p53–p21 pathway and induce inhibition of G_1-phase Cdk activities. Immunoblotting of p53 and p21 in 2008 cells that had been treated with 0.5, 1.5, and 3.0 μM of DAP for 12, 24, 36, and 48 h showed that higher concentrations of DAP delayed or attenuated the p53 and p21 induction (Figure 11c). Such an inverse dose-effect of DAP on the p53–p21 induction was not observed in any other cell lines used in this study (data not shown). Consistent with these results, G_1-phase progression and G_1-phase Cdk activities were not inhibited in 2008 cells at any of these DAP concentrations (data not shown). These results further indicated that amount of p21 induction determines all of DAP-induced G_1-phase checkpoint responses.

Discussion

We previously established that DAP treatment of ovarian cancer cells primarily blocks G_1-phase progression and that DAP-induced G_1-phase arrest requires

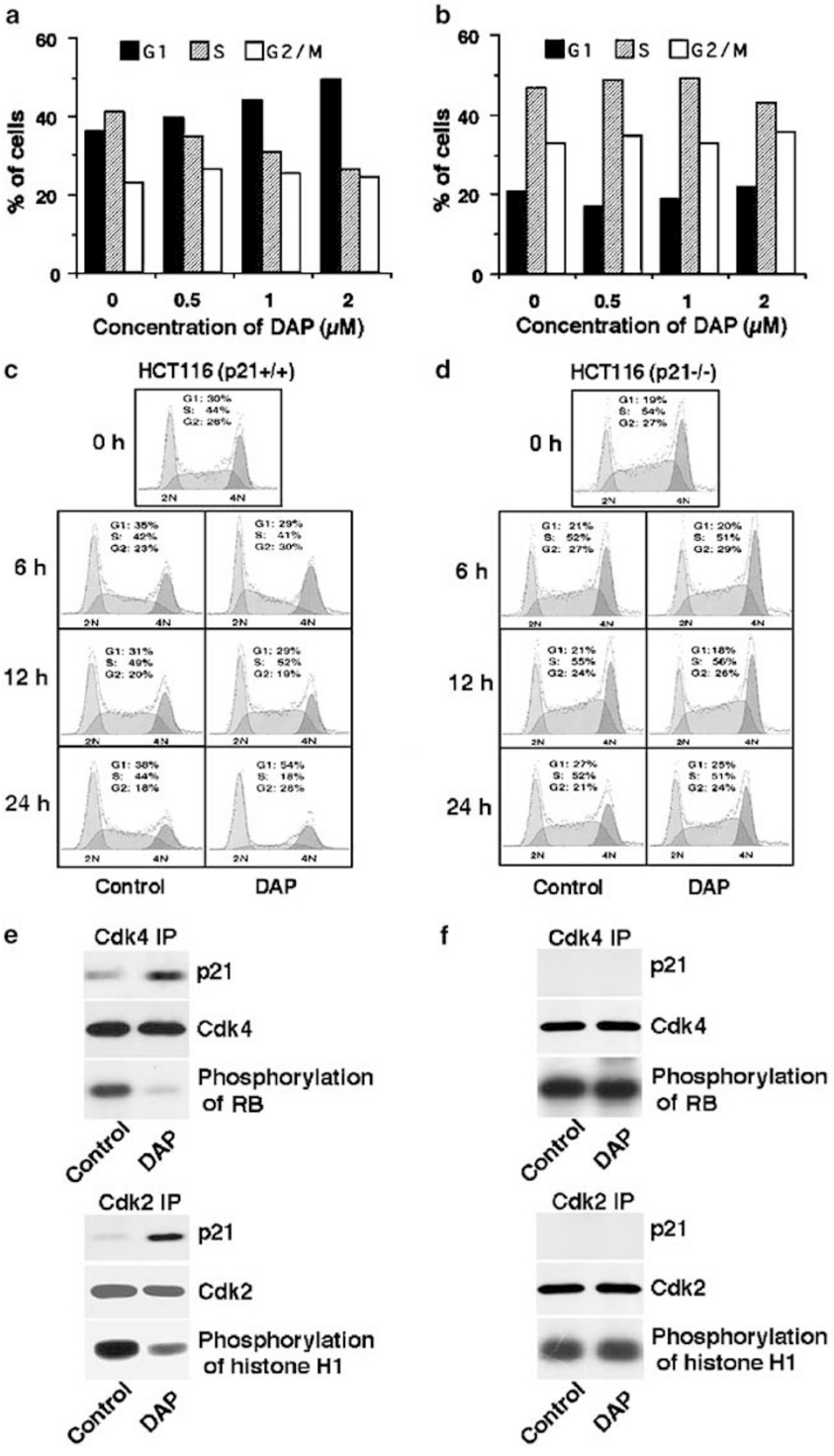

Figure 8 p21 induction is required for DAP-induced inhibition of Cdk4 and Cdk2 activities. (**a, b**) p21-containing HCT-116 cells (**a**) or p21-deleted HCT-116 cells (**b**) were treated with indicated concentrations of DAP for 24 h, and the cell cycle distribution was determined by FACS. (**c, d**) p21-containing HCT-116 cells (**c**) or p21-deleted HCT-116 cells (**d**) were treated with 2 μM DAP for 6, 12, or 24 h, and the cell cycle distribution was determined by FACS. (**e, f**) p21-containing HCT-116 cells (**e**) or p21-deleted HCT-116 cells (**f**) were treated with 2 μM DAP for 24 h, and Cdk4 or Cdk2 was immunoprecipitated from crude lysates of DAP-treated or control cells. While the Cdk4 immunocomplex was immunoblotted for p21 and Cdk4 and assayed for RB kinase activity, the Cdk2 immunocomplex was immunoblotted for p21 and Cdk2 and assayed for histone H1 kinase activity

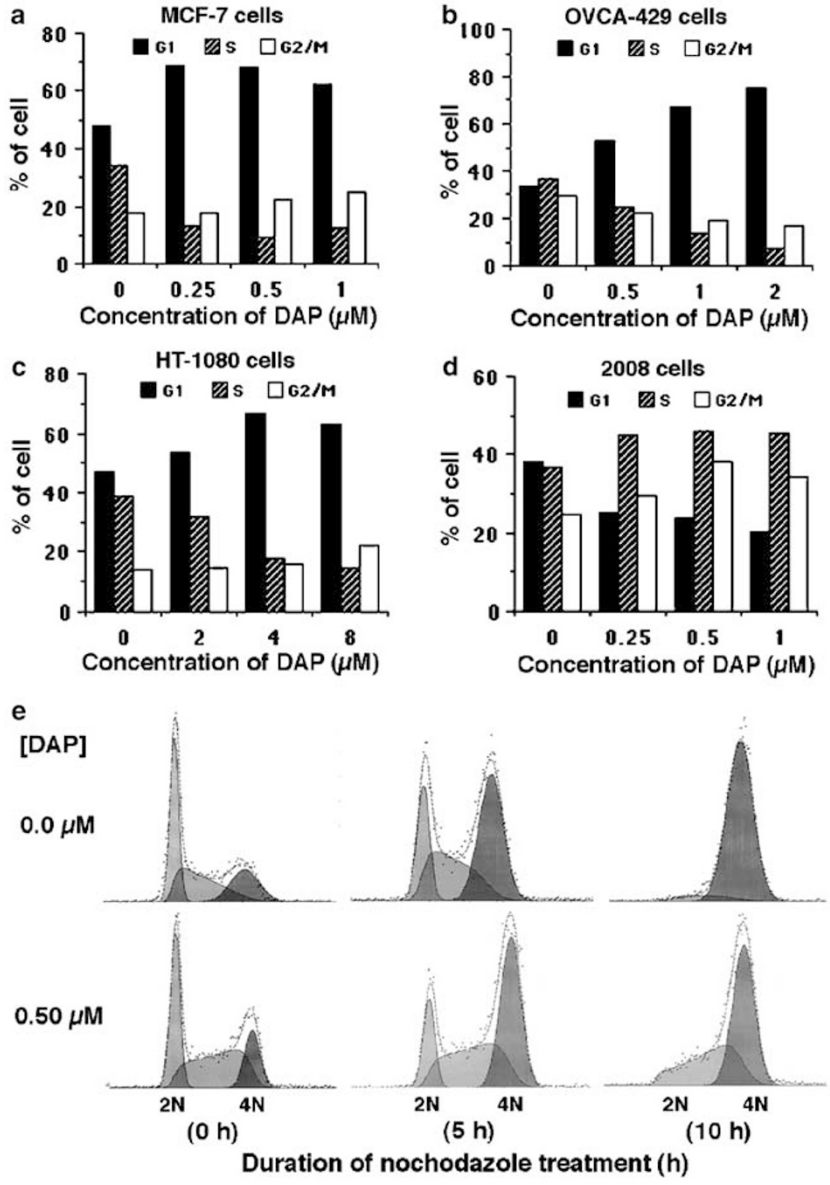

Figure 9 Cell cycle effects of DAP treatment on additional cancer cell lines. (**a–d**) MCF-7 cells (**a**), OVCA-429 cells (**b**), HT-1080 cells (**c**), and 2008 cells (**d**) were treated with indicated concentrations of DAP for 24 h, and the cell cycle distribution was determined by FACS. (**e**) After 2008 cells were cultured in the presence or absence of 0.5 μM DAP for 24 h, nocodazole was added at 0.8 μg/ml and cells were further cultured for 10 h. The cell cycle distribution was determined at 0, 5, and 10 h after the nocodazole addition

wild-type p53 activity (Hagopian *et al*., 1999). Moreover, DAP-induced G_1-phase arrest is associated with inhibition of both Cdk4 and Cdk2 activities and followed by induction of apoptosis (Kuang *et al*., 2001). To further characterize the effect of DAP action on the G_1-phase checkpoint, this study was designed to identify the mechanisms that are responsible for DAP-induced G_1-phase Cdk inhibition. To achieve this goal, we initially utilized A2780 cells as the model system to investigate systematically which of the known mechanisms are involved in the DAP-induced Cdk inhibition. Our data showed that DAP treatment does not cause degradation of G_1-phase cyclins, inhibition of their association with their partner Cdks, induction of inhibitory phosphorylation of G_1-phase Cdks, or inhibition of activating phosphorylation of G_1-phase Cdks. In contrast, DAP treatment elevates the level of p53 protein, p21 transcript, and p21 protein in a sequential manner. The induced p21 quantitatively binds to both the Cdk4/cyclin D and Cdk2/cyclin E complexes and inhibits both their kinase activities. Inhibition of protein synthesis by cycloheximide at the time of p21 induction prevents DAP-induced inhibition of both Cdk4 and Cdk2 activities. These results indicated that p21 induction by p53 is responsible for DAP-induced inhibition of both Cdk4 and Cdk2 activities. To further test this conclusion, we utilized a pair of colon cancer cell lines that differ only in the p21

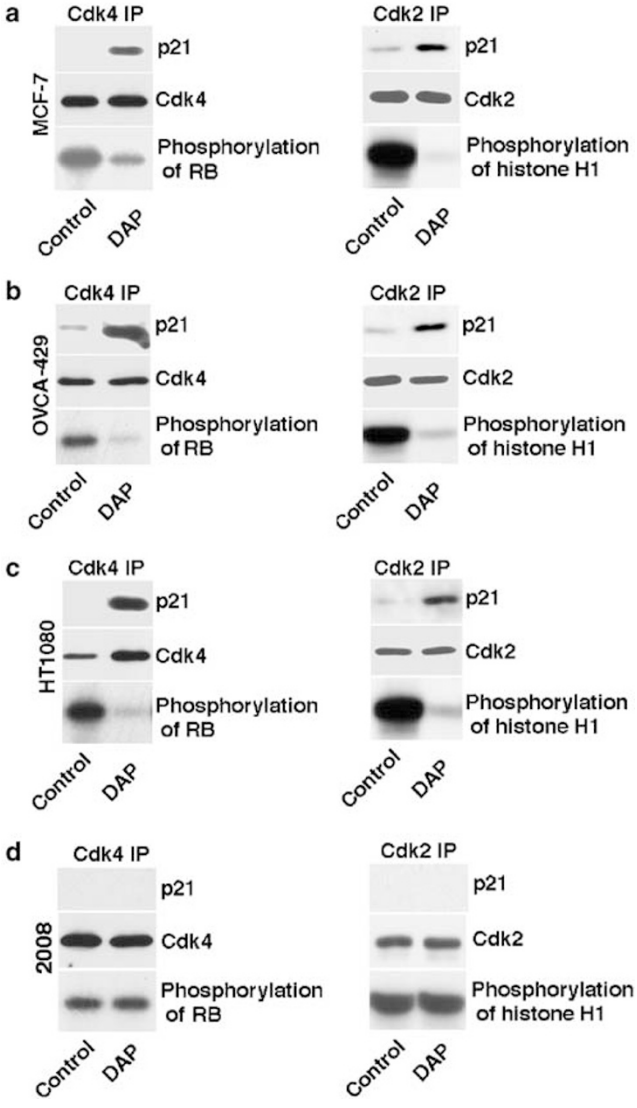

Figure 10 Cdk effects of DAP treatment on additional cancer cell lines. MCF-7 cells (**a**), OVCA-429 cells (**b**), HT-1080 cells (**c**), or 2008 cells (**d**) were treated with 0.5, 2, 4, or 0.5 μM DAP for 24 h, and total proteins were extracted from DAP-treated or control cells. Cdk4 or Cdk2 was then immunoprecipitated (IP) from each pair of crude cell lysates. While the Cdk4 immunocomplex was immunoblotted for p21 and Cdk4 and assayed for RB kinase activity, the Cdk2 immunocomplex was immunoblotted for p21 and Cdk2 and assayed for histone H1 kinase activity

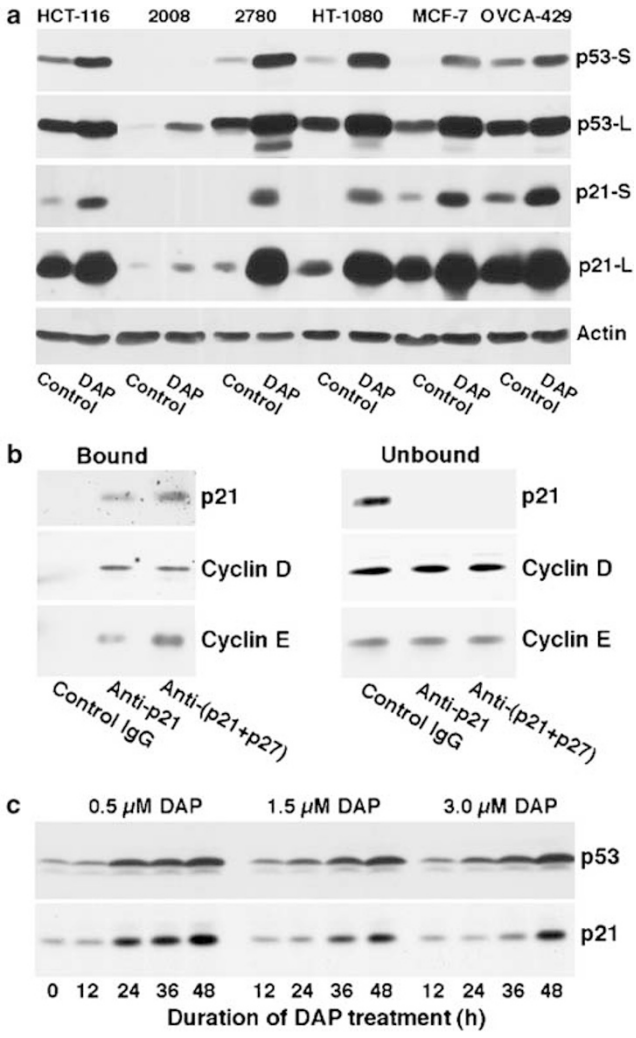

Figure 11 Lack of G_1-phase Cdk effects of DAP treatment on 2008 cells is associated with dramatically reduced levels of p53 and p21. (**a**) 2008, A2780, HCT-116, HT-1080, MCF-7, or OVCA-429 cells were treated with 0.5, 0.6, 2, 4, 0.5, or 2 μM DAP for 24 h, and total proteins were extracted from DAP-treated or control cells. Same amounts of proteins were taken from different cell lysates and immunoblotted for p53, p21, and actin. Two different exposures of p53 and p21 blots are shown. L indicates long exposure and S indicates short exposure. (**b**) 2008 cells were treated with 0.5 μM DAP for 24 h, and total proteins were extracted from DAP-treated or control cells. After crude cell lysates were immunoprecipitated either with anti-p21 antibodies alone or with both anti-p21 and anti-p27 antibodies, bound and unbound fractions of the proteins from the immunoprecipitation were immunoblotted for p21, cyclin D, and cyclin E. (**c**) 2008 cells were treated with 0.5, 1.5, or 3.0 μM DAP for 48 h, and total proteins were extracted at 12-h intervals. Same amounts of proteins from different cell lysates were immunoblotted for p53 and p21

gene status and determined requirement of p21 induction for the DAP-induced G_1-phase checkpoint responses. Our data showed that p21 deletion eliminates both DAP-induced G_1-phase arrest and inhibition of Cdk4 and Cdk2 activities, confirming our conclusion. We also utilized four additional cancer cell lines that contain functional p53 and p21 and examined effects of DAP treatment on the cell cycle progression, G_1-phase Cdk activities, and induction of p53 and p21. We found that whereas DAP treatment of breast cancer MCF-7 cells, fibrosarcoma HT-1080 cells, or ovarian cancer OVCA-429 cells results in G_1-phase arrest and inhibition of both Cdk4 and Cdk2 activities, it produces none of these effects in ovarian cancer 2008 cells. In line with this contrast, basal-level and DAP-induced expression of p53 and p21 are greatly attenuated in 2008 cells as compared to other cell lines examined, and DAP-induced p21 in 2008 cells binds to only small portions of the Cdk4 and Cdk2 complexes. These findings provided further support to the conclusion that p21 induction by

p53 is responsible for DAP-induced inhibition of both Cdk4 and Cdk2 activities.

Prior to this study, it has been generally assumed that p21 induction by p53 during DNA damage-induced G_1-phase checkpoint responses only inhibits Cdk2 activity and that Cdk4 inhibition is achieved through a different mechanism (Boulaire *et al*., 2000; Bartek and Lukas, 2001a, b; Agami and Bernards, 2002). This thinking is based on lack of sufficient evidence to establish that p21 induction by p53 following DNA damage can inhibit Cdk4 activity and presence of indirect evidence suggesting that induced p21 in DNA-damaged cells may not inhibit Cdk4 activity. Previous studies have provided three lines of evidence, which together establish the model that activation of the p53–p21 pathway during DNA damage-induced G_1-phase checkpoint responses leads to inhibition of Cdk2 activity. First, p21 induction following DNA damage greatly increases the level of p21 in the Cdk2 complex (Dulic *et al*., 1994; el-Deiry *et al*., 1994; Petrocelli *et al*., 1996; Poon *et al*., 1996). Second, p21 that is released from the inhibited Cdk2 complex or inhibited cell lysates by heat treatment can inhibit the kinase activity of the active Cdk2 complex isolated from cycling cells *in vitro* (Dulic *et al*., 1994). Third, p21 gene knockout in mouse embryonic fibroblast cells abolishes the p53-dependent Cdk2 inhibition induced by DNA damage (Brugarolas *et al*., 1995, 1999; Deng *et al*., 1995). However, previous studies have not obtained similar evidence for inhibition of Cdk4 activity by p21 induction. With regard to p21 binding, although there are reports that p21 induction following DNA damage significantly increases the level of p21 in both the Cdk4 and Cdk2 complexes (Petrocelli *et al*., 1996), there is also evidence that induced p21 in DNA-damaged cells preferentially binds to the Cdk2 complex (Poon *et al*., 1996; Brugarolas *et al*., 1999). In terms of Cdk4 inhibition, none of previous studies have tested whether p21 that is isolated from DNA-damaged cells can inhibit the activity of the active Cdk4 complex isolated from cycling cells. However, one previous study showed that addition of recombinant p21 to lysates of cycling cells at physiologically relevant levels inhibits Cdk2 but not Cdk4 activity (Poon *et al*., 1996). Moreover, there were reports that p21 and p27 facilitate assembly of the Cdk4/cyclin D and thus promote its activity (LaBaer *et al*., 1997; Cheng *et al*., 1999; Sherr and Roberts, 1999). In addition, p27 is preferentially associated with the Cdk4 complex in mammalian proliferating fibroblast cells (Polyak *et al*., 1994; Toyoshima and Hunter, 1994), and redistribution of p27 from the Cdk4 to Cdk2 complex is associated with inhibition of Cdk2 activity under various growth inhibitory conditions (Poon *et al*., 1996; Jiang *et al*., 1998; Sherr and Roberts, 1999; Lenferink *et al*., 2001; Nahum *et al*., 2001). Together, these observations led to the speculation that p21 binding to the Cdk4 complex does not inhibit Cdk4 activity during DNA damage-induced G_1-phase checkpoint responses. Finally, genetic studies have not been performed to address whether p21 gene deletion abolishes Cdk4 inhibition in a p53-dependent G_1-phase checkpoint response induced by DNA damage. In this study, we made the first attempt to utilize concurrently the three previously utilized criteria to evaluate the role of p21 induction in both Cdk4 and Cdk2 inhibitions during a DNA damage-induced G_1-phase checkpoint response. Our findings provide the first solid evidence that activation of the p53–p21 pathway following DNA damage can inhibit both Cdk4 and Cdk2 activities in the absence of activating other known mechanisms of the Cdk inhibition and challenge the current thinking that p21 induction by p53 specifically inhibits Cdk2 activity.

To account for previously reported preferential binding of p21 to the Cdk2 complex upon UV irradiation of mammalian fibroblast cells (Poon *et al*., 1996), it is possible that majority of the pre-existing Cdk4 complex has already been associated with and inactivated by p27 (Polyak *et al*., 1994; Toyoshima and Hunter, 1994; Bagui *et al*., 2000; Bagui *et al*., 2003) and thus does not need p21 binding for inactivation. In this context, it is noteworthy that DAP treatment of A2780 cells induces a great increase in the amount of the Cdk4 and Cdk2 complexes (Figure 2), making the pre-existing p27-containing Cdk4 complex quantitatively less significant in the final population of the inhibited Cdk4 complex. This may be why we did not detect preferential binding of p21 to the Cdk2 complex in our experimental systems. To explain previously reported preferential inhibition of Cdk2 activity by addition of recombinant p21 to lysates of cycling cells (Poon *et al*., 1996), it is possible that there is a difference between recombinant p21 and induced p21 in DNA-damaged cells or p21 preferentially binds to the Cdk2 activity under conditions of insufficient amounts of p21 induction. To avoid these complications, we utilized p21 that was released from DAP-treated cell lysates and treated the Cdk4 and Cdk2 complexes isolated from control cells in parallel. Under these experimental conditions, less p21 was actually required to inhibit Cdk2 activity than Cdk4 activity (data not shown), consistent with the notion that majority of the pre-existing Cdk4 complex has already been associated with and inactivated by p27.

Previous studies have identified two mechanisms that can lead to Cdk4 inhibition during DNA damage-induced G_1-phase checkpoint responses. One is Cdk4 tyrosine phosphorylation (Terada *et al*., 1995) and the other is cyclin D degradation (Poon *et al*., 1996; Agami and Bernards, 2000). However, due to reasons described earlier, it seems unlikely that these are among the common mechanisms responsible for Cdk4 inhibition during DNA damage-induced G_1-phase checkpoint responses. Since activation of the p53–p21 pathway is a frequent G_1-phase checkpoint event following both high and low levels of DNA damage, the findings in this study suggest the possibility that activation of this pathway is a common mechanism responsible for Cdk4 inhibition during DNA damage-induced G_1-phase checkpoint responses.

Previous studies showed that cisplatin treatment of human cancer cells usually causes cell cycle effects in 8–12 h at low toxic concentrations (Goldwasser *et al*., 1996; Pestell *et al*., 2000). Consistent with these previous

observations, treatment of A2780 cells with 1.0 μm cisplatin induces cell cycle arrest with a 6-h delay (unpublished results). However, our results showed that low effective concentrations of DAP inhibited G1-phase progression with a 18-h delay in A2780 cells (Figure 1a) and 24-h delay in HCT-116 cells (Figure 8c), which were much longer than the delays in cisplatin-induced cell cycle effects. The mechanism of DAP-induced Cdk4 and Cdk2 inhibitions may explain this difference. Among different checkpoint control mechanisms that target Cdk activities, induction of Cdk tyrosine phosphorylation or cyclin degradation belongs to the type that does not involve *de novo* RNA and protein syntheses and thus induces acute cell cycle effects (Bartek and Lukas, 2001a, b). In contrast, the p53–p21 pathway belongs to the type that requires *de novo* RNA and protein syntheses and thus produces slow cell cycle effects. Since cisplatin treatment of human cancer cells activates both the ATR–Chk1 pathway that induces Cdk tyrosine phosphorylation (Zhao and Piwnica-Worms, 2001; Eastman *et al*., 2002a) and the p53–p21 pathway (Burger *et al*., 1998; Zamble *et al*., 1998; Ueno *et al*., 1999), it produces both acute and slow cell cycle effects. Because DAP activates the p53–p21 pathway without activating the ATR–Chk1 pathway, it selectively induces slow cell cycle effects. This unique feature of DAP raises two interesting issues that warrant further studies. One is why DAP specifically activates the p53–p21 pathway and the other is whether having delayed cell cycle effects only protects cells from apoptosis.

Materials and methods

Cell culture, drug treatment and FACS

DAP was synthesized and purified as described previously (Khokhar *et al*., 1997). Cycloheximide and nocodazole were purchased from Sigma Chemical Company. Ovarian cancer A2780 and 2008 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Fibrosarcoma HT-1080 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. Breast cancer MCF-7 cells and ovarian cancer OVCA-429 cells were cultured in MEM medium supplemented with 10% fetal bovine serum. Colon cancer HCT-116 cells were cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum. For DAP treatment of A2780 cells, 1–1.3 $\times 10^6$ cells were plated in 100-mm culture dishes and grown for at least 24 h before the cultures were changed into fresh medium containing 0.6 μM DAP. The cells were then further grown for indicated lengths of time and collected for determination of the cell cycle distribution by FACS as previously described (Kuang *et al*., 2001) or protein extraction. DAP treatment of other cell lines was also conducted at subconfluence.

Preparation of crude cell lysates, immunoblotting, immunoprecipitation, and kinase assays

Preparation of crude cell lysates and immunoblotting were performed as previously reported (Kuang *et al*., 2001). Primary antibodies used to immunoblot Cdk4 (MS-299), Cdk2 (MS-617), cyclin A (MS-384), and p27 (MS-256) were purchased from Neomarkers Inc. (Fremont, CA, USA). Primary antibodies used to immunoblot cyclin D (sc-718), cyclin E (sc-247), and p21 (sc-6246) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody used to immunoblot actin (A5060) was purchased from Sigma Chemical Company. PY99 (sc-7020) and 4G10 (05-321) anti-phosphotyrosine antibodies were obtained from Santa Cruz Biotechnology and Upstate Biotechnology (Lake Placid, NY, USA), respectively. Primary antibodies used to immunoblot Thr160-phosphorylated Cdk2, Ser345-phosphorylated Chk1, or Thr68-phosphorylated Chk2 were obtained from Cell Signaling (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection kit to develop the immunoblots were purchased from Amersham Pharmacia (Piscataway, NJ, USA).

Immunoprecipitation was performed with immobilized antibodies as previously described (Kuang *et al*., 2001). Antibodies used to immunoprecipitate Cdk4 (sc-260), Cdk2 (sc-163), cyclin D (sc-718), cyclin B1 (sc-245), p21 (sc-397), and p27 (sc-528) were purchased from Santa Cruz Biotechnology. The antibody used to immunoprecipitate cyclin E (PC438) was purchased from Oncogene Research Products (Boston, MA, USA). Retinoblastoma mutated protein (Rb) or histone 1 (H1) kinase activity of the Cdk4 or Cdk2 complexes was assayed as previously described (Kuang *et al*., 2001).

Dephosphorylation of Cdk4 and Cdc2 with Cdc25

Xenopus Cdc25C cDNA, which was kindly provided by Dr Nebreda (Perdiguero *et al*., 2003), was used as the template to PCR-amplify the coding region for amino residues 205–550 of the protein. The forward primer was 5′-CGGGATCCGCTT-TACCCACTGTTACTG-3′, which contained a *Bam*HI site. The reverse primer was 5′-TTAGCGGCCGCTTATGCGGG-CAATCTGTT-3′, which contained a *Not*I site. After the PCR product was digested with both *Bam*HI and *Not*I, it was subcloned into pGEX4T3 vector (Amersham) after the GST-coding sequence. The GST fusion protein (GST-Cdc25) was then produced in BL21 *Escherichia coli* cells and affinity-purified as previously described (Che *et al*., 1999). The Cdc2 or Cdk4 complex was immunoprecipitated from control- or DAP-treated cell lysates with anti-cyclin B1 or anti-Cdk4 antibodies as described earlier. Dephosphorylation of Cdc2 or Cdk4 with GST-Cdc25 was performed by incubation of 10 μl of the Cdk4 or cyclin B1 immunocomplex with 0.2 μg GST-Cdc25 in 20 μl dilution buffer (150 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT in 50 mM Tris-HCl, pH 7.4) at room temperature for 45 min. The treated Cdk4 or cyclin B immunocomplexes were assayed for RB or histone H1 kinase activity as previously described (Kuang *et al*., 2001).

Northern blot analysis

The total RNA was extracted from A2780 cells by using Trizol (GIBCO BRL, Grand Island, NY, USA) according to the manufacturer's instruction. After the sample was separated by electrophoresis through a 0.8% agarose gel containing 1.5% v/v formaldehyde, RNAs were transferred to nylon membranes, hybridized with ^{32}P-labeled p21 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA at 42°C for 18 h, and washed with 0.1 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate for at least 30 min. Blots were then exposed to X-ray film for 6–8 h.

In vitro *inhibition of Cdk4 and Cdk2 activities by heat-released p21 from DAP-treated cells*

After A2780 cells were cultured in the presence or absence of 0.6 μM DAP for 24 h, crude lysates of 10 mg/ml protein were

prepared from both DAP-treated or control cells. From the control cell lysate, the active Cdk4 or Cdk2 complex was immunoprecipitated with anti-Cdk4 or anti-Cdk2 antibodies (0.5 μg antibodies per 25 μl cell lysates) immobilized onto protein-G agarose. The immunocomplexes were washed twice with cell extraction buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 100 mM NaCl, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1 μg/ml each of leupeptin, pepstatin, and chymostatin). To obtain DAP-induced p21, the DAP-treated cell lysate was heated at 100°C for 5 min and the sample that contained 5% of the total protein was recovered after centrifugation at 13 000 *g* for 10 min. Depletion of p27 or both p21 and p27 from the soluble fraction was achieved by immunoabsorption with anti-p27 antibodies alone or a combination of anti-p21 and anti-p27 antibodies. For treatment of the Cdk4 or Cdk2 complex, 2.5 or 16 μl of the original DAP-treated cell lysate or the soluble fraction of the boiled DAP-treated cell lysate was diluted to a final volume of 50 μl with the cell extraction buffer that was supplemented with different amounts of BSA to even the final protein concentration to 10 mg/ml. Different samples were then incubated with the Cdk4 or Cdk2 complex isolated from the control cell lysate at room temperature for 45 min. After the beads were washed, they were either immunoblotted for cyclin D (for the Cdk4 complex) and cyclin E (for the Cdk2 complex) or measured for the kinase activity as described above.

Acknowledgements
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