The cyclin-dependent kinase inhibitor p21 is required for a sustained G2 arrest after activation of the DNA damage checkpoint. Here we have addressed the mechanism by which p21 can contribute to this arrest in G2. We show that p21 blocks the activating phosphorylation of Cdc2 on Thr161. p21 does not interfere with the dephosphorylation of two inhibitory phosphorylation sites on Cdc2, Thr14 and Tyr15, indicating that p21 targets a different event in Cdc2 activation as the well described DNA damage checkpoint pathway involving Chk1 and Cdc25C. Taken together our data show that a cell is equipped with at least two independent pathways to ensure efficient inhibition of Cdc2 activity in response to DNA damage, influencing both positive and negative regulatory phosphorylation events on Cdc2.

Onset of mitosis is brought about by the activation of the cyclin-dependent kinase (cdk) Cdc2. Cdc2 activation requires binding to cyclin B and phosphorylation of a conserved threonine residue (Thr161) present in the so-called T-loop of Cdc2. Phosphorylation of Thr161 is mediated by the cdk-activating kinase (CAK) (reviewed in Ref. 2), consisting of CDK7, cyclin H, and MAT1 (3–5). In addition, the cyclin B-Cdc2 complex can be inhibited by phosphorylation of Cdc2 at two negative regulatory sites, namely Thr14 and Tyr15 (2). Phosphorylation of these sites is regulated by the inhibitory Wee1 protein kinase and the activating Cdc25 protein phosphatases (1, 6). Moreover, cyclin B-Cdc2 complexes are subject to active nuclear import and export, and during G2, export of the nuclear export protein Crm1 occurs at a very high rate, so that cyclin B is mostly cytoplasmic (7, 8). It is not until the G2/M transition that the cyclin B-Cdc2 complexes are translocated to the nucleus, where they can phosphorylate their targets and initiate mitosis.

Cyclin B-Cdc2 complexes are maintained in an inactive state until the end of G2 by phosphorylation of the Thr14/Tyr15 residues. Around the time of nuclear translocation of the complex, these residues are dephosphorylated, resulting in the formation of an active cyclin B-Cdc2 complex (2). As mentioned, this dephosphorylation occurs by a Cdc25 protein phosphatase. Three Cdc25 family members have been identified to date, A, B and C, the last one being the active one at the onset of mitosis. The activity of Cdc25C itself can be enhanced through phosphorylation by cyclin B-Cdc2 (9, 10). Therefore, activation of cyclin B-Cdc2 has been proposed to result in an autocatalytic feedback loop to ensure rapid activation of these complexes at the G2/M transition (9, 10).

Activation of cyclin B-Cdc2 complexes is delayed if the G2 DNA damage checkpoint is activated. This is in part the result of phosphorylation of Cdc2 at Thr14 and Tyr15 (2). DNA damage prevents the dephosphorylation of Thr14/Tyr15 sites of Cdc2 through activation of Chk1, a component of the DNA damage checkpoint pathway (11, 12). Chk1 is activated upon binding and phosphorylates Cdc25C on Ser216, and this results in binding of Cdc25C to the 14-3-3 family of proteins (13, 14). Binding to 14-3-3 prevents nuclear translocation of Cdc25C, thus inhibiting access of Cdc25C to Cdc2 (15).

In addition to regulating Thr14/Tyr15 phosphorylation, the DNA damage checkpoint also appears to affect nuclear translocation of cyclin B (16, 17). Recently, it was shown that sequestration of cyclin B-Cdc2 complexes in the cytoplasm in response to DNA damage requires 14-3-3 protein, which itself is a p53-responsive gene that can arrest cells in G2 when overexpressed (19). This indicates that expression of 14-3-3 protein is upregulated upon irradiation in a p53-dependent manner and contributes to the G2 block induced by DNA damage.

Next to induction of 14-3-3 protein, p53 also drives accumulation of the cdk inhibitor p21 (20). Although initially thought to be involved solely in the DNA damage checkpoint in G1, p21 was later shown to play a crucial role in the G2 DNA damage checkpoint as well (21). Here we have addressed the mechanism by which p21 can contribute to this arrest in G2. We show that p21 targets an event in cyclin B-Cdc2 activation different from that targeted by the pathways described previously. Thus, multiple phosphorylation events on Cdc2 are affected by DNA damage via independent pathways.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Synchronization, and Reagents—UTA21.15 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1 μg/ml tetracycline, and p21 induction was performed as described (22). For synchronization at the G1/S transition cells were synchronized by a double thymidine block. To this end, cells were treated with thymidine (2.5 mm, Sigma) for 16 h, after which the thymidine was washed away, and 12 h after the release thymidine was added back to the cultures for another 16 h. This protocol reproducibly resulted in synchronization of >80% of cells in the G1 compartment, and >80% of the cells re-enter the cell cycle upon release in control as well as p21-induced cultures. Cells were arrested at the metaphase/
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anaphase transition of the M phase by treating the cells with nocodazole (250 ng/ml; Sigma) for 16 h.

Protein A/G plus agarose, the mouse monoclonal antibodies against cyclin B1 (GNS1) and Cdc2 (clone 17), and polyclonal anti-cdk2 (M2), anti-p21 (C-19), and anti-Cdc25C (C-20) were all purchased from Santa Cruz. Polyclonal phosphospecific Cdc2-Tyr15 antibody was obtained from New England BioLabs, and Plk1 was immunoprecipitated with rabbit polyclonal anti-Plk (Zymed Laboratories Inc.). Fluorescein isothiocyanate-conjugated anti-BrdUrd antibody was obtained from Becton Dickinson. Histone H1 and sequence grade trypsin were obtained from Roche Molecular Biochemicals. Unphosphorylated α-casein and propidium iodide were purchased from Sigma.

Cell Cycle Analysis—DNA profiles were obtained by staining cells with propidium iodide and flow cytometric analysis as described (22). For BrdUrd pulse-chase analysis, cells were pulsed with 1 μM BrdUrd for 10 min at 37°C, chased with fresh medium without BrdUrd, and prepared for bivariate flow cytometry as described (22). To determine mitotic indices, cells were grown on coverslips and synchronized by thymidine and p21 expression was induced by removal of tetracycline using anti-cyclin B1 (GNS1, Santa Cruz) and counterstained with DAPI to analyze the nuclear morphology. Immunofluorescence was recorded using confocal laser fluorescence microscopy.

Cdk-mediated Phosphorylation of Cdc2, in Vivo Phosphate Labeling, and Peptide Mapping—Recombinant Cdk was prepared by infection of SF9 insect cells with baculoviruses encoding cyclin H, hemagglutinin-tagged cdkt2 and MAT1 (25). 48 h post-infection cells were lysed in 50 mM dithiothreitol, 5 mM MgCl₂, 20 mM Tris, pH 7.5, 50 mM KCl, supplemented with 1 μg/ml leupeptin, 2.5 μg/ml aprotinin, 10 μM β-glycerophosphate, and 0.5 mM phenylmethylsulfonyl fluoride. Cell lysate containing recombinant Cdk was incubated for 15 min at 30°C with immunoprecipitates of cyclin B prepared from UTA21.15 cells. Mock-infected insect cells were used as control and contained no detectable Cdk activity. Cyclin B immunocomplexes were then washed with 150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40 supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2.5 μg/ml aprotinin, 10 μM β-glycerophosphate, and 5 mM NaF, and kinase assays were then performed as described (23). For in vivo phosphate labeling, UTA21.15 cells were synchronized with thymidine and p21 expression was induced by removal of tetracycline or treatment with camptothecin as described above. Cells were released from the block in the presence of nocodazole to analyze accumulation of mitotic cells. At the indicated time points after the release coverslips were processed for immunofluorescence using anti-cyclin B1 (GNS1, Santa Cruz) and counterstained with DAPI to analyze the nuclear morphology. Immunofluorescence was recorded using confocal laser fluorescence microscopy.

Western Blotting, Immunoprecipitation, and In Vitro Kinase Reactions—Western blotting was performed as described (22). Immunoprecipitation and in vitro kinase assays of cyclin A and cdk2 were performed as described (22). In vitro kinase assays of cyclin B and analysis of the phosphorylation state of Cdc2 complexed to cyclin B were performed as described (23). Plk1 in vitro kinase assays were performed as described, using casein as substrate (24).

Subcellular Localization of Cyclin B1—Cells were plated on coverslips and synchronized by thymidine treatment and induced to express p21 as described above. Cells were released from the block in the presence of nocodazole to analyze accumulation of mitotic cells. At the indicated time points after the release coverslips were processed for immunofluorescence using anti-cyclin B1 (GNS1, Santa Cruz) and counterstained with DAPI to analyze the nuclear morphology. Immunofluorescence was recorded using confocal laser fluorescence microscopy.

CAK-mediated Phosphorylation of Cdc2, in Vivo Phosphate Labeling, and Peptide Mapping—Recombinant CAK was prepared by infection of SF9 insect cells with baculoviruses encoding cyclin H, hemagglutinin-tagged cdkt2 and MAT1 (25). 48 h post-infection cells were lysed in 5 mM dithiothreitol, 5 mM MgCl₂, 20 mM Tris, pH 7.5, 50 mM KCl, supplemented with 1 μg/ml leupeptin, 2.5 μg/ml aprotinin, 10 μM β-glycerophosphate, and 0.5 mM phenylmethylsulfonyl fluoride. Cell lysate containing recombinant CAK was incubated for 15 min at 30°C with immunoprecipitates of cyclin B prepared from UTA21.15 cells. Mock-infected insect cells were used as control and contained no detectable Cdk activity. Cyclin B immunocomplexes were then washed with 150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40 supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2.5 μg/ml aprotinin, 10 μM β-glycerophosphate, and 5 mM NaF, and kinase assays were then performed as described (23). For in vivo phosphate labeling, UTA21.15 cells were synchronized with thymidine and p21 expression was induced by removal of tetracycline or treatment with camptothecin as described above. Cells were released from the block, and 18 h after release cells were labeled with 0.5 Ci/ml [³²P]orthophosphate (Amersham Pharmacia Biotech) for 4 h, cells were lysed, and [³²P]labeled cyclin B-Cdk2 complexes were immunoprecipitated. Immunocomplexes were separated on SDS-polyacrylamide gels and blotted to polyvinylidene difluoride membrane (Immobilon). Labeled Cdc2 was cut out from the membrane and treated as described previously for peptide mapping (26).
RESULTS

**p21 Induces a Sustained G₂ Arrest**—We have investigated the effect of p21 at the G₂/M transition by making use of a U2OS osteosarcoma cell line expressing p21 from a tetracycline-regulatable expression vector (UTA21.15 cells (22)). Induction of p21 from the tetracycline-repressible promoter in UTA21.15 cells results in levels of p21 expression that are very similar to the level of expression of p21 reached when these cells are treated with ionizing radiation or DNA damaging agents (22). Therefore, this system lends itself well to study the effects of p21 that would occur after DNA damage. As a first approach to study the effects of p21 during G₂/M in more detail, cells in S phase were labeled with [3H]thymidine, and cell cycle progression of BrdUrd-positive cells was monitored for several hours. Expression of p21 results in a block somewhere in G₂, or M, because less than 20% of the cells have entered G₂ after 16 h (Fig. 1a), whereas most cells in the control cultures have. Similar results were obtained when cells were released from a double thymidine block after induction of p21. Again, almost all cells that re-enter the cell cycle are eventually arrested with a 4 N DNA content, whereas the control cultures continue into the next G₁ phase (Fig. 1b).

To distinguish whether these cells were in G₂ or M phase, we analyzed the appearance of mitotic cells in these same samples. Again, cells were synchronized using thymidine and released from this block after induction of p21. After release, nocodazole was added to examine accumulation of mitotic cells. Control cultures start to enter mitosis about 12 h after release from the thymidine block, and at 21 h ~50% of these cells have entered mitosis (Fig. 1c). In contrast, we could detect very little mitotic cells in the cultures induced to express p21 (Fig. 1c), although FACS analysis confirmed that a similar percentage of cells had reached a DNA content of 4 N (data not shown). These data clearly demonstrate that p21 can block cells in G₂.

To confirm that p21 is induced similarly by the tetracycline-regulatable system as it is induced by DNA damage in thymidine-blocked cells, we analyzed p21 expression by Western blotting (Fig. 1d). Treatment of thymidine-blocked cells with the DNA damaging agent camptothecin resulted in an efficient induction of p21 and p21 levels remained elevated throughout the release. Similar effects on p21 expression were observed upon removal of tetracycline from the medium (Fig. 1d). In control cultures only a very low induction of p21 protein was seen (Fig. 1d), which is consistent with the described bimodal peak of p21 mRNA in G₁ and G₂/M (27).

**p21 Inhibits Activation of Cyclin B-Cdc2 Complexes**—Previously, we had shown that induction of p21 in asynchronic cultures of UTA21.15 cells results in inhibition of cyclin B-associated kinase activity (22). However, under these conditions cells will arrest both in the G₁ phase and G₂/M phase of the cell cycle, making it hard to draw any conclusions on G₂ phase-specific effects of p21. Therefore, we decided to study the effect of p21 when induced in cells synchronized at the G₁/S transition using a thymidine synchronization protocol as described above. Induction of p21 after synchronization with thymidine results in a very effective inhibition of cyclin-dependent kinase activity of cyclin E/cdk2 (not shown) and cyclin A-cdk2 complexes (Fig. 1e). However, progression through S phase was not affected in these cells (Fig. 1f and data not shown), demonstrating that cells do not require high cyclin/cdk activity to complete S phase. This finding is in line with the fact that
neutralizing antibodies against cyclin A can block S phase when injected during G1, but are no longer effective when injected in cells synchronized at G1/S with hydroxyurea (28). As is shown in Fig. 2a, we can detect a wave of cyclin B-associated kinase activity around 15–18 h after release from the thymidine block in control cultures, consistent with the timing of mitosis under these conditions (Fig. 1, b and c). However, this increase of cyclin B-associated kinase activity is not seen in cells induced to express p21 (Fig. 2a). To analyze this effect of p21 on cyclin B-associated kinase activity in more detail, we added nocodazole to the cultures after release from the thymidine block. This way mitotic exit is blocked, and the breakdown of cyclin B is prevented. Under these conditions, cyclin B-associated kinase activity continues to rise in the control cultures up to 21 h, but very little cyclin B-associated kinase activity is detected in p21-expressing cells (Fig. 2b). This absence of cyclin B-associated kinase activity is not a mere consequence of inhibition of cyclin B synthesis, because induction of cyclin B protein expression was not affected by p21 (Fig. 2c).

p21 Inhibits Thr161 Phosphorylation of Cdc2—One obvious possibility by which p21 could prevent activation of cyclin B-Cdc2 complexes is through stoichiometric binding to these complexes. To investigate what fraction of cyclin B-Cdc2 complexes associated with p21 under these circumstances, we metabolically labeled UTA21.15 cells with [35S]methionine and immunoprecipitated p21 or cyclin B. Indeed, a considerable amount of cyclin B com immunoprecipitated with p21 in p21-induced cultures (Fig. 3a), but only a fraction of the total cyclin B pool could be immunoprecipitated. Also, we only found a minor decrease (>25%) in the amount of cyclin B present in a cell lysate depleted of p21 by sequential immunoprecipitation (data not shown). Thus, p21 is able to bind cyclin B, but this binding does not appear to be stoichiometric. This is consistent with previous reports that p21 has a low affinity for cyclin B-Cdc2 complexes (29–31). However, despite the relatively low amounts of p21 present in the cyclin B immunocomplexes, the kinase activity of these complexes is inhibited to an extent that cannot be explained by mere binding of p21 to these complexes. Therefore, we decided to investigate other determinants of cyclin B-Cdc2 activity, to understand which other factors could contribute to the observed inhibition.

It has been described that p21 and family members can interfere with CAK-mediated phosphorylation of cdk2 on the Thr160 residue (32, 33), a phosphorylation event required for activation of cdk2 (reviewed in Ref. 2). To investigate whether p21 could prevent cyclin B-Cdc2 activation also by interference with CAK-mediated phosphorylation of Cdc2 on the Thr161 residue, we tested the ability of recombinant trimeric CAK to in vitro activate cyclin B-Cdc2 complexes isolated at different time points after release from a thymidine block. The inactive cyclin B-Cdc2 complexes from p21-expressing cells were efficiently reactivated by CAK (Fig. 3b) to levels comparable with cyclin B-Cdc2 complexes from control cultures. In vitro activity of cyclin B-Cdc2 complexes from control cultures was only poorly elevated by pretreatment with CAK. This indicates that cyclin B-Cdc2 complexes are poorly phosphorylated by CAK in cells induced to express p21, as compared with control cultures.

Thus, our data suggest that p21 blocks activation of cyclin B-Cdc2 complexes by preventing Thr161 phosphorylation of the Cdc2 subunit by CAK. To confirm this, we performed phosphopeptide analysis of in vitro phosphorylated Cdc2. UTA21.15 cells were labeled with [32P]orthophosphate at 18 h after release from a thymidine block. Western blot analysis of cyclin B immunoprecipitates prepared at this time point indicated that a significant fraction of Cdc2 was dephosphorylated on the Thr14/Tyr15 residues in control cultures and in UTA21.15 cells induced to express p21 (Fig. 3c, right panel). In contrast, Thr14/Tyr15 dephosphorylation was completely blocked by the DNA damaging agent camptothecin in these cells (Fig. 3c, right panel). Although the upper band, representing Thr14/Tyr15-phosphorylated Cdc2 was efficiently phospholabeled in vivo, the lower band contained relatively little [32P] (Fig. 3c, left panel). This suggests that the Thr161 site is inefficiently labeled under these circumstances, consistent with previous reports (34). Nevertheless, we did observe a clear inhibition in the phosphorylation of the Thr161 site in the cultures induced to express p21 (Fig. 3, c and d). A similar inhibition in Thr161 phosphorylation was seen in cells treated with the DNA damaging agent camptothecin (Fig. 3d), demonstrating that inhibition of Thr161 phosphorylation is not limited to our inducible p21 system but also occurs as a general response to DNA damage.

p21 Does Not Inhibit Thr14/Tyr15 Dephosphorylation of Cdc2—DNA damage is thought to prevent the G2/M transition by two mechanisms. One is the Chk1-mediated inhibition of cyclin B-Cdc2 complexes (11, 12), and the other is by affecting the nuclear translocation of cyclin B (16, 17). Our results show that by inducing p21, the DNA damage pathway triggers another mechanism of cyclin B-Cdc2 inactivation. We next questioned whether p21 could also affect other control mechanisms of cyclin B-Cdc2 activation. To analyze the status of cyclin B-Cdc2 complexes in more detail, we performed immunoblots of cyclin B immunoprecipitates isolated at different time points after release from a thymidine block. Complex formation with Cdc2 occurred normal as judged from immunoblots of cyclin B immunoprecipitates probed with anti-Cdc2 (Figs. 3c and 4a). Activation of the cyclin B-Cdc2 complex requires dephosphorylation of Cdc2 on its Thr14/Tyr15 residues by Cdc25C. This dephosphorylation has been shown to result in a Cdc2 conformation with higher electrophoretic mobility (35). We can detect this faster migrating form of Cdc2 at 15–18 h in control cultures released from a thymidine block, after which this form of Cdc2 disappears again (Fig. 4a). This timing of Thr14/Tyr15 dephosphorylation coincides with the time points at which we could detect cyclin B-associated kinase activity (Fig. 2a). Thr14/Tyr15 dephosphorylation of Cdc2 occurs at similar time points in the cultures induced to express p21 (Fig. 4a), but this form of Cdc2 does not disappear, consistent with our finding that these cells do not pass through mitosis. Again, we chose to confirm these findings in a similar experimental setup, but with the addition of nocodazole during the release. Thr14/Tyr15 dephos-
phosphorylation of Cdc2 is clearly not inhibited by p21 as judged by the shift in electrophoretic mobility of Cdc2 (Fig. 4b). Using a phospho-specific antibody raised against Tyr15-phosphorylated Cdc2, we were able to confirm that the upper band represents Tyr15-phosphorylated Cdc2 (data not shown). Moreover, using this same antibody we could show that Tyr15-phosphorylated Cdc2 disappeared from control and p21-expressing cultures with similar kinetics (Fig. 4c). Therefore, cells expressing high levels of p21 seem to arrest in G2 without preventing complex formation and Thr14/Tyr15 dephosphorylation of cyclin B-Cdc2.

Thr14/Tyr15 Dephosphorylation Does Not Depend on Positive Feedback—From the experiment shown in Fig. 4, it is clear that Thr14/Tyr15 dephosphorylation occurs normal in cultures induced to express p21, although the kinase activity of cyclin B-Cdc2 complexes is inhibited. This would argue against the requirement of an autocatalytic feedback loop between Cdc25C and cyclin B-Cdc2 as has been described by others (9, 10). We therefore analyzed the phosphorylation state of Cdc25C in the presence of high levels of p21. Interestingly, p21 can prevent phosphorylation of Cdc25C, because the mobility shift of Cdc25C associated with phosphorylation by cyclin B-Cdc2 (9, 10) is blocked in cells expressing high levels of p21, as compared with control cultures (Fig. 5a). Thus, although a very tight correlation between the mobility shift and activity of Cdc25C has been reported (36, 37), our data would suggest that Cdc25C activation occurs in the absence of such a mobility shift. Other kinases have been reported to activate Cdc25C, most prominently, the polo family of protein kinases (38, 39). We therefore investigated the effect of p21 on the activation of the polo-like kinase Plk1 in these cells. Indeed, p21 did not prevent activation of Plk1 (Fig. 5b), indicating that Plk-mediated activation of Cdc25C can occur normally under these circumstances.

p21 Does Not Inhibit Nuclear Translocation of Cyclin B—As described above, DNA damage was shown to affect translocation of cyclin B-Cdc2 complexes from the cytoplasm to the nucleus, which normally takes place at the G2/M transition (7, 8). We therefore investigated whether p21 could prevent nuclear translocation of cyclin B. To this end we analyzed the subcellular distribution of cyclin B in cells at different time points after release from a thymidine block in the presence of nocodazole as described above. In control cultures we found that translocation of cyclin B to the nucleus was tightly linked with the onset of mitosis, as determined by chromosomal condensation (judged by DAPI staining; not shown) (Fig. 6). Translocation of cyclin B to the nucleus was not inhibited in cells induced to express p21 but appeared to be accelerated compared with control cultures (Fig. 6). However, nuclear translocation of cyclin B and entry into mitosis were clearly uncoupled, because almost all of the nuclei positive for cyclin B remained in interphase in cultures induced to express p21, as judged by DAPI staining (not shown).

We have shown that induction of p21 expression after initiation of DNA replication results in a G2 arrest. This p21-induced block in G2 is maintained for up to 24 h, indicating that p21 can sustain a G2 arrest for a significant amount of time. By utilizing p21-inducible cells, we have been able to untangle several events that normally occur very rapidly prior to mitosis. We show that in the absence of cyclin/cdk activity, cells can progress to several points in the cell cycle while cyclin-B-Cdc2 complexes are translocated to the nucleus, and Cdc2 is dephosphorylated on Thr14/Tyr15. Our data indicate these events do not depend on prior activation of cyclin A-cdk2 or cyclin A-Cdc2 complexes. Also, these events do not require activation of the cyclin B-Cdc2 complexes themselves. Similarly, Plk1 is normally activated, and the fact that Cdc2 is dephosphorylated on Thr14/Tyr15 would suggest that Cdc25C is also normally activated. This latter observation argues against a requirement for a positive feedback loop between cyclin B-Cdc2 complexes and the activating Cdc25C phosphatase (9, 10). Formally, we cannot exclude the possibility that a phosphatase other than Cdc25C can dephosphorylate Cdc2 under these conditions. Assuming Cdc25C is the phosphatase responsible for Thr14/Tyr15 dephosphorylation of Cdc2, our data would suggest that Plk-mediated phosphorylation of Cdc25C is sufficient for full activation of Cdc25C phosphatase activity. In addition, our data show that cells can progress through S phase into G2, with severely reduced cyclin-dependent kinase activity, because cyclin E-, cyclin A- and cyclin B-associated kinase activities are efficiently inhibited by p21.

Cyclin B-Cdc2 complexes have a relatively low affinity for
p21 when compared with the other cyclin-cdk complexes (29). Indeed, we could only detect low amounts of p21 in cyclin B immunoprecipitates, whereas the cyclin B-associated kinase activity was fully inhibited after inducing p21. This indicates that stoichiometric binding of p21 to these complexes could not be solely responsible for the observed inhibition of the kinase activity. However, we cannot rule out the possibility that p21 efficiently binds and directly inhibits active cyclin B-Cdc2 complexes in vivo. Nevertheless, incubation with recombinant CAK provides a very efficient means to reactivate the cyclin B-Cdc2 complexes, indicating that the major restriction on the kinase activity is the lack of Thr161 phosphorylation. The presence of low levels of p21 in cyclin B immunocomplexes could prevent CAK from complete reactivation of cyclin B-Cdc2 complexes. Inhibition of CAK-mediated phosphorylation of cdk2 by p21 has been shown to occur previously for cdk2 (32, 33). This block in T160 phosphorylation of cdk2 is not due to inhibition of CAK activity in the cell, but p21 binding to cyclin/cdk complexes is thought to block the access of CAK to the activating phosphorylation site. The data presented here indicate that a similar mechanism applies to inhibition of cyclin B-Cdc2 complexes by p21.

Our results demonstrate that induction of p21 after DNA damage contributes to inactivation of cyclin B-Cdc2 complexes via a mechanism that is distinct from what has been described so far for DNA damage-induced inhibition of cyclin B-Cdc2. Based on our data we would like to propose that a cell has the capacity to block Cdc2 activation by at least two independent pathways upon activation of the G2 DNA damage checkpoint (Fig. 7). The first pathway, comprising Chk-1 and Cdc25C, blocks activation of Cdc2 through retention of Thr14/Tyr15 phosphorylation. The second pathway, comprising p53 and p21, inhibits Cdc2 by blocking the activating Thr161 phosphorylation. Because activation of Chk1 is a much more rapid response than induction of p21 expression, one could hypothesize that the Chk1/Cdc25C pathway is responsible for immediate inhibition of existing cyclin B-Cdc2 complexes, whereas the p21/CAK pathway would ensure a more sustained arrest. Such a model would be consistent with the findings of Bunz et al. (21), who showed that expression of p53 and p21 is not required for the initial arrest, but both proteins are essential to sustain the G2 arrest in response to DNA damage. As such, the p53/p21 pathway results in an important, independent contribution to inactivation of Cdc2, and lack of this response in certain tumor cells is expected to increase their sensitivity to DNA damaging agents.

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