Cyclin A–associated kinase activity is needed for paclitaxel sensitivity

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Abstract
Cyclin A–associated kinases, such as cyclin-dependent kinase 2 (CDK2), participate in regulating cellular progression from G1 to G2, and CDK2 has also been implicated in the transition to mitosis. The antitumor properties of CDK inhibitors, alone or in combination with taxanes, are currently being examined in clinical trials. Here, we examined whether the activity of kinases associated with cyclin A (such as CDK2) is important in determining cellular sensitivity to paclitaxel, a taxane and mitotic inhibitor used in chemotherapy for breast and ovarian cancer. We used adenoviral suppression or overexpression to manipulate the expression of CDK2 and cyclin A in one breast cancer and three ovarian cancer cell lines with different sensitivities to paclitaxel and assessed protein expression, kinase activity, cell cycle distribution, and sensitivity to paclitaxel. Transfection of a dominant-negative (DN)-CDK2 evoked resistance to paclitaxel by preventing cellular progression to mitosis through loss of CDK1 activity. Reexpression of wild-type CDK2 in DN-CDK2–transfected cancer cells restored CDK2 activity but not paclitaxel sensitivity. However, expression of cyclin A in DN-CDK2–transfected cells restored their sensitivity to paclitaxel. Although CDK2 activity was not directly involved in paclitaxel sensitivity, cyclin A–associated kinases did up-regulate CDK1 via phosphorylation. We conclude that cyclin A–associated kinase activity is required for these cells to enter mitosis and undergo paclitaxel-induced cell death.

Introduction
Cell cycle progression is regulated by several cyclin-dependent kinases (CDK). One such kinase, CDK2, participates in regulation of the G1-S phase transition through its association with cyclin E; CDK2 also functions in the S-G2 progression through its association with cyclins A and E (1–3). Overexpression of cyclin E or cyclin A in solid tumors, including breast cancer, has been linked with adverse outcomes (4–7). Cyclin E in particular seems to be a powerful predictor of poor outcome in breast cancer (8).

Because CDK2 activity may be related to patient outcome, CDK2 has been an attractive target for the development of small-molecule chemical inhibitors. CDK inhibitors, such as flavopiridol, UCN-01 (7-hydroxystaur- osporine), CYC202 (R-roscovitine), and BMS-387032, all of which have some anti-CDK2 activity (9, 10), are being investigated in clinical trials as single agents and in combination with taxane chemotherapy (9, 10).

Taxanes, such as paclitaxel or docetaxel, are an indispensable component of current chemotherapy regimens for breast and ovarian cancer (11–13). Taxanes act by artificially stabilizing microtubules (14, 15). The microtubule dynamics are particularly critical during mitosis as they are responsible for the capture and alignment of chromosomes at metaphase and their subsequent separation into two daughter cells at anaphase. The disruption of microtubule dynamics by taxanes leads first to mitotic arrest, resulting in a sustained or transient cell cycle block and eventually to cell death from apoptosis or mitotic catastrophe, a type of cell death caused by an aberrant exit from mitosis (16–19). Many investigators have presumed a link between cell cycle progression and sensitivity to paclitaxel (20). Surprisingly, very few studies have dealt with this question directly. Indeed, known mechanisms of taxane resistance include overexpression of P-glycoprotein, tubulin mutations, and abnormal expression of Bcl-2 (21–23), but the role of cell cycle progression itself in taxane resistance has not been defined at the molecular level.

We and others found that activation of the spindle assembly checkpoint, which monitors the progression of mitosis, is required for paclitaxel sensitivity (24, 25). Activation of this checkpoint involves activation of CDK1/cyclin complexes, which are an important component of the G2-M phase transition (26, 27). Overexpression taxane chemotherapy with any drug targeting cyclin A–associated kinases (e.g., pure CDK2 inhibitors) should be done with caution, if at all, because of the potential for enhancing taxane resistance. [Mol Cancer Ther 2005; 4(7):1039–46]
of ErbB2 receptor tyrosine kinase, which confers resistance to paclitaxel, was shown to directly inhibit CDK1 and thus block progression of cells into M phase, suggesting that entry into mitosis is a key factor in taxane sensitivity (28).

Interestingly, CDK2 has also been implicated in the entry into mitosis through its regulation of CDK1 activity (29, 30). However, whether the activity of cyclin A–associated kinases (such as CDK2) contributes directly to paclitaxel resistance is completely unknown. This novel question needs to be answered because CDK inhibitors are currently being developed as potential therapeutic agents to be used in combination with paclitaxel. Here, we hypothesized that suppression of cyclin A–associated kinases leads to paclitaxel resistance. Specifically, we aimed to clarify the role of CDK2, a major cyclin A–associated kinase, in the M-phase transition and in the sensitivity of breast cancer and ovarian cancer cells to paclitaxel.

Materials and Methods

Cells andViruses

The cell lines studied were MDA-MB-468 breast cancer cells and 2774, OVCA433, and OVTOKO ovarian cancer cells, which display different levels of CDK2 activity and paclitaxel sensitivity. The MDA-MB-468 and OVCA433 cells were obtained from the M. D. Anderson Breast Cancer Research Program Core Laboratory Cell Line Depository, and the 2774 and OVTOKO cells were obtained from the Tottori University School of Medicine. Cells were grown in DMEM/F12 medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum, 0.1 mmol/L MEM nonessential amino acid solution, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 2 mmol/L L-glutamine (all from Invitrogen, Carlsbad, CA). Recombinant adenoviruses containing cDNA for a dominant-negative (DN)-CDK2 construct or for cyclin A were created as described elsewhere (31). An adenovirus containing cDNA for wild-type CDK2 was kindly provided by Dr. Joseph R. Nevins (Duke University Medical Center, Durham, NC; ref. 32). A control vector, Ad.mock, contained E1B and lacked E1A and E3 (33). All of the recombinant adenoviruses (Ad.mock, Ad.DN-CDK2, Ad.WT-CDK2, and Ad.cyclin A) were replication defective, all lacked E1A, and all were grown in the permissive 293 cell line and purified with a BD Adeno-X virus purification kit (BD Biosciences, San Jose, CA). Viral titers were determined with a BD Adeno-X rapid titer kit (BD Biosciences) and expressed as infectious units (ifu) per milliliter. The titer for the Ad.mock construct was 3.5 × 10^10 ifu/mL; that for Ad.DN-CDK2 was 1.9 × 10^10 ifu/mL; for Ad.WT-CDK2, 3.2 × 10^10 ifu/mL; and for Ad.cyclin A, 2.6 × 10^10 ifu/mL. All other reagents, including paclitaxel, were purchased from Sigma Chemical (St. Louis, MO).

Immunoprecipitation, Western Blotting, and Kinase Activity Assays

To evaluate CDK2 and CDK1 activity, cells were lysed for 30 minutes at 4°C in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Nonidet P40, 25 mmol/L sodium fluoride, 200 μmol/L sodium orthovanadate, 5 mmol/L β-glycerophosphate, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 5 μg/mL phenylmethylsulfonyl fluoride. Protein extracts (100–250 μg per sample) were precleared to reduce nonspecific adsorption to immunoprecipitates by a 1-hour incubation with 15 μL of a Protein G Plus A-agarose solution (Oncogene Research Products, Boston, MA). The precleared lysates were then subjected to immunoprecipitation with 30 μL of protein G/A-agarose beads bound to either 2 μg of D-12, an anti-CDK2 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or a mixed mouse monoclonal antibody to cyclin B (Upstate Biotechnology, Inc., Lake Placid, NY). After extensive washes, the immunoprecipitates were suspended in 20 μL of kinase buffer [50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 2.5 mmol/L EGTA, 1 mmol/L DTT, 0.1% Triton X-100, 100 μmol/L sodium fluoride, and 100 μmol/L sodium orthovanadate] supplemented with 20 μL of unlabeled ATP, 50 μg/mL of histone H1 as a substrate, and 2 μCi of [γ-32P]ATP per sample, and incubated for 30 minutes at 30°C. The reactions were terminated by the addition of 5 μL of 5× sample buffer, and the labeled proteins were resolved by SDS-PAGE and detected by autoradiography. Kinase activity was determined by using the NIH Image program to compare the density of the bands formed with that of cells infected with Ad.mock. Protein levels were measured by resuspended the immunoprecipitates in 25 μL of sample buffer and incubating them for 10 minutes at 37°C. Protein extracts were separated by SDS-PAGE and electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Protein levels were then analyzed with the rabbit polyclonal antibodies H298 (for CDK2) or H432 (for cyclin A) or H433 (for cyclin B; all from Santa Cruz Biotechnology) and a peroxidase-conjugated secondary antibody and detected with an electrochemiluminescence system (Amersham Biosciences, Piscataway, NJ). Anti-CDK1 rabbit antibody and agarose-conjugated p13suc1 were purchased from Upstate Biotechnology. Thr161-phosphorylated CDK1 rabbit antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti–β-actin mouse antibody was purchased from Sigma Chemical.

Cell Viability

Cell viability was determined with a Premix WST-1 Cell Proliferation Assay system (Takara Mirus Bio, Madison, WI) in which the tetrazolium salt WST-1 in formazan is cleaved by mitochondrial dehydrogenases in viable cells (34). Cells were plated at 4,000 to 6,000 cells/well in 96-well plates and exposed to various concentrations of paclitaxel (Sigma Chemical) for 72 hours. Each concentration was tested in triplicate wells. After the incubation period, the WST-1 reagent was added to each well, and absorbance at 450 nm was measured 1 hour later. The IC50 and IC25 values were calculated as the concentrations of paclitaxel that caused 50% and 75% growth inhibition of treated cells relative to growth of cells that were infected but not treated with paclitaxel.
Bromodeoxyuridine Pulse-Chase Assay

Cell cycle phase transitions were assessed with a bromodeoxyuridine (BrdUrd) pulse-chase assay as described previously (35). Briefly, cells were infected with the viral constructs and 24 hours later were pulse-labeled with 10 μmol/L BrdUrd for 30 minutes at 37°C. At the end of the pulse-labeling period, cells were washed with PBS and either harvested immediately by trypsinization or cultured in fresh medium. Cells were fixed with 70% ethanol and stored at 4°C before analysis. The fixed cells were washed with PBS and incubated with 2 N HCl, and either harvested immediately by trypsinization or cultured in fresh medium. The pellet was washed with PBS and incubated with anti-BrdUrd antibody conjugated with FITC (1:20; BD Biosciences) for 1 hour at room temperature. At the end of the incubation, RNase A was added to a final concentration of 250 μg/mL and the mixture was incubated for 15 minutes at room temperature. The nuclei were counterstained with 5 μg/mL propidium iodide for 15 minutes, and stained cells were analyzed by flow cytometry. Data from a minimum of 5,000 nuclei were acquired and displayed as dual-parameter propidium iodide versus log-FITC histograms, and the fraction of BrdUrd-positive cells (right) was calculated by CellQuest software (BD Biosciences).

Results

Suppressing CDK2 Increases Resistance to Paclitaxel

First, to examine whether a decrease in CDK2 activity would affect sensitivity to paclitaxel (a mitotic inhibitor used in chemotherapy for breast and ovarian cancer), we infected the following cell lines with Ad.DN-CDK2: MDA-MB-468 breast cancer cells [with 10 multiplicity of infection (MOI); Fig. 1A], 2774 ovarian cancer cells [with 10 MOI; Fig. 1B], OVCA433 ovarian cancer cells [with 25 MOI; Fig. 1C], and OVTOKO ovarian cancer cells [with 50 MOI; Fig. 1D]; infection was followed by paclitaxel treatment and a cell viability assay. Introduction of the DN-CDK2 reduced the sensitivity of all four cell lines. As expected, infection with DN-CDK2 reduced cellular CDK2 kinase activity by 80% at 24 hours (data not shown) and by 90% at 48 hours relative to that of mock-infected MDA-MB-468 cells (Fig. 1F) and other cell lines (data not shown).

Restoring CDK2 Activity Does Not Restore Paclitaxel Sensitivity in Cancer Cells

To examine whether this increased resistance to paclitaxel depended directly on the reduction in CDK2 kinase activity, we simultaneously infected MDA-MB-468 cells with 50 MOI of Ad.WT-CDK2 and 10 MOI of Ad.DN-CDK2 to allow recovery of CDK2 activity. Coexpression of WT-CDK2 resulted in recovery of cellular CDK2 activity to 90% of the level of mock-infected cells at 24 hours (data not shown) and to 80% at 48 hours after infection (Fig. 1F). However, sensitivity to paclitaxel was not restored despite recovery of CDK2 activity (Fig. 1E).

We next used BrdUrd incorporation to examine the effects of DN-CDK2 on cell cycle progression in the presence or absence of WT-CDK2 (Fig. 2). In the control (mock infection) condition, 46% of the cells incorporated BrdUrd, which means about half of the cells underwent DNA synthesis (S phase). After 20 hours, the majority of these BrdUrd-labeled cells had moved to a region of higher DNA content (G2-M phase) and then on to one of less DNA content after division (G1 phase), which reflected the cells having completed one cycle (Fig. 2A–C).

Expression of DN-CDK2 (Fig. 2D–F) suppressed BrdUrd incorporation by 15% compared with that of mock-infected cells (46% versus 31%; Fig. 2D) and slowed the progression of BrdUrd-labeled cells from G1-S compared with the control, causing S-phase delay or G2-M phase arrest (Fig. 2F). Coexpression of WT-CDK2 with DN-CDK2 (Fig. 2G–I) restored BrdUrd incorporation to the same level as control (46% versus 48%; Fig. 2G) and allowed most of the cells to progress through S phase, but the cells arrested at G2-M (Fig. 2I). Expression of DN-CDK2, alone or with WT-CDK2, increased the proportion of cells G2-M phase at 20 hours compared with the mock-infection condition (Fig. 2C, F, and I; inserts).

Further, the mitotic index (the percentage of cells in mitosis divided by that of cells in all other phases) of cells expressing DN-CDK2, with or without WT-CDK2, was lower than that of the mock-infected cells at 20 hours and even at 48 hours after the chase. In summary, BrdUrd and mitotic index studies suggest that DN-CDK2–transfected cells undergo G2, but despite recovery of CDK2 activity by WT-CDK2 do not reach M phase.

Cyclin A–Associated Kinases Are Essential for Paclitaxel Sensitivity

Because cyclin A is known to regulate the S to G2-M phase transitions by different mechanisms including regulation of CDK2, we coexpressed cyclin A and DN-CDK2 in the Ad.DN-CDK2–infected cells and found that this combination restored CDK2 activity at 48 hours (Fig. 1F). Further, paclitaxel sensitivity was restored to some extent relative to the sensitivity of the mock-infected cells (the IC50 was 2.5 nmol/L for mock-infected cells versus 5 nmol/L for cyclin A + DN-CDK2–coinfected cells; the IC25 was 8 nmol/L for mock-infected cells versus > 100 nmol/L for cyclin A + DN-CDK2–coinfected cells; Fig. 1E). Coexpression of WT-CDK2 and cyclin A (at 25 MOI of each) in the Ad.DN-CDK2–infected cells increased CDK2 kinase activity by four to five times compared with mock-transfected cells (Fig. 1F). However, the coexpression of WT-CDK2 and cyclin A restored sensitivity to paclitaxel but did not enhance it beyond that of cells infected with Ad.cyclin A (Fig. 1F). We also examined whether CDK2 kinase activity changed after paclitaxel treatment to rule out the possibility that paclitaxel did not change CDK2 activity at all. However, the CDK2 kinase activity levels were similar at 48 hours (and 24 hours; data not shown) after infection in cells not treated with paclitaxel (Fig. 1F, left) and in cells treated with paclitaxel (Fig. 1F, right).
Overexpression of CDK2 Does Not Enhance Sensitivity to Paclitaxel

We next examined the effect of overexpressing WT-CDK2 or cyclin A, separately at 50 MOI each or together at 25 MOI each, on paclitaxel sensitivity in MDA-MB-468 cells (Fig. 3). Overexpression of these molecules increased CDK2 activity by a factor of 2 to 5 at 24 hours after infection (Fig. 3A), an increase similar to that induced by coexpression of WT-CDK2 and cyclin A in the Ad.DN-CDK2–infected cells (Fig. 1F). However, any increase in sensitivity to paclitaxel was quite limited, as reflected by the IC50 values (50 nmol/L for the mock infection condition, 10 nmol/L for WT-CDK2, 10 nmol/L for cyclin A, and 10 nmol/L for WT-CDK2 plus cyclin A; Fig. 3B) and certainly did not reflect the extent of the increase in CDK2 activity.

Cyclin A, but not CDK2, Restores CDK1 Activity and Paclitaxel Sensitivity

We next examined how cyclin A–associated kinase could restore paclitaxel sensitivity by examining whether cyclin A–associated kinase activity would lead to activation of cyclin B–associated kinase (CDK1), which is essential for spindle assembly checkpoint activation and paclitaxel sensitivity (24). To answer this question, we infected cells with Ad.DN-CDK2 in combination with WT-CDK2 or cyclin A and measured CDK1 activity. DN-CDK2 expression decreased CDK1 activity (Fig. 4) as it did for CDK2 activity (Fig. 1F). Attempts to restore CDK2 activity by coexpressing WT-CDK2 with DN-CDK2 did not restore CDK1 activity (Fig. 4) but restored CDK2 activity (Fig. 1F). However, coexpressing cyclin A with DN-CDK2, with or without WT-CDK2, restored CDK1 activity (Fig. 4). In addition, in mock-infected cells, the CDK1 kinase activity at 48 hours after paclitaxel treatment was similar to that of cells not treated with paclitaxel; for cells treated with cyclin A, however, the CDK1 kinase activity was increased in the paclitaxel condition compared with no-paclitaxel conditions (Fig. 4, compare left with right) because paclitaxel caused M arrest. Finally, the increase in CDK1 activity paralleled an enhancement in paclitaxel sensitivity from the infection of cyclin A (Fig. 1E). The observed increases in CDK1/cyclin B activity after paclitaxel exposure in cells infected with cyclin A suggest that a cyclin A–associated kinase other than CDK2 activated the CDK1, a finding consistent with our recent report (24).

Further, to determine whether DN-CDK2 could be influencing paclitaxel resistance by trapping cyclin A bound to CDK1, we used agarose-conjugated p13suc1 (which specifically binds CDK1) to immunoprecipitate CDK1 proteins from cells infected with Ad.DN-CDK2 or Ad.WT-CDK2 and measured levels of cyclin A bound to CDK1. Infection with DN-CDK2 resulted in a 40% decrease in cyclin A relative to that of mock-infected cells (Fig. 4B). However, overexpressing CDK2 via infection with Ad.WT-CDK2 also trapped cyclin A bound to CDK1 at roughly the same levels as in the DN-CDK2 condition (Fig. 4B). Nevertheless, although WT-CDK2 could trap cyclin A (bound to CDK1), overexpression of WT-CDK2 alone did

Figure 1. Changes in CDK2 kinase activity and paclitaxel sensitivity by coexpression of WT-CDK2 or cyclin A with DN-CDK2. MDA-MB-468 (A), 2774 (B), OVCA433 (C), and OVTOKO (D) cells were infected with Ad.mock or Ad.DN-CDK2 (at 10 MOI in A and B; at 25 MOI in C; and at 50 MOI in D). At 24 h after infection, the cells were cultured with the indicated concentrations of paclitaxel for an additional 48 h. E and F, MDA-MB-468 cells were infected with the indicated combinations of Ad.mock, Ad.DN-CDK2, Ad.WT-CDK2, and Ad.cyclin A. At 24 h after infection, the cells were cultured in the presence of paclitaxel for an additional 24 h (E) or 48 h (F). Cell viability was determined with a WST-1 assay and expressed relative to the viability of control cells that had been infected with the same adenoviruses but not treated with paclitaxel; CDK2 activity and immunoprecipitated CDK2 levels were measured in the presence or absence of 100 nmol/L paclitaxel. The ratio refers to CDK2 kinase activity compared with that of the mock-infected cells.
not increase paclitaxel resistance (Fig. 3B). These findings suggest that sequestration of cyclin A from CDK1 by DN-CDK2 was not the major cause of inducing paclitaxel resistance.

**Cyclin A–Associated Kinase Restores Thr161-Phosphorylated CDK1**

Finally, to explore how CDK1 activity was inhibited by DN-CDK2 and restored by coexpression of cyclin A in MDA-MB-468 cells, we analyzed the phosphorylation status of CDK1 under the various test conditions. Phosphorylation of CDK1 at Thr161 is necessary for its catalytic activity (36). We found that 10 MOI of Ad.DN-CDK2 reduced the levels of phosphorylated CDK1 protein (which migrates more slowly than does unphosphorylated CDK on SDS-PAGE) and that coexpression of 50 MOI of Ad.cyclin A, but not 50 MOI of Ad.WT-CDK2, restored the levels of phosphorylated CDK1 at 24 and 48 hours (Fig. 5). Cyclin B levels, on the other hand, were not affected by coexpression of WT-CDK2 or cyclin A with DN-CDK2 at 24 or 48 h (Fig 5). Collectively, these findings suggest that a cyclin A–associated kinase is needed to activate CDK1.

**Discussion**

A variety of evidence has implicated CDK activation in the apoptosis induced by chemotherapeutic agents. In one study, CDK1 activity was transiently increased by the topoisomerase inhibitors camptothecin and etoposide (37). Another group found that the cyclin A–associated kinases CDK1 and CDK2 were activated during apoptosis induced by inhibitors of protein kinases or phosphatases such as staurosporine, 6-dimethylaminopurine, or okadaic acid (38). Apoptosis induced by UCN-01 (7-hydroxystaurosporine), another protein kinase inhibitor, was also shown to depend on the activation of CDK1 and CDK2 (39). Conversely, expression of DN mutants of CDKs (40–42), endogenous CDK inhibitors such as p21waf1/cip1 or p27kip1, or exogenous CDK inhibitors, such as olomoucine, have all been shown to suppress apoptosis (41, 43–45). However, no studies have directly addressed whether modification of CDKs, especially CDK2, affects paclitaxel sensitivity.

In this study, we showed that paclitaxel had antitumor activity only in cells that could progress to M phase. Cells that enter premitosis but cannot activate CDK1 will not be efficiently killed by paclitaxel. We also found that progression to M phase requires the activity of a cyclin A–associated kinase. CDK2, one such kinase, allowed these cells to progress from G1 to pre-G2 but was not sufficient to induce paclitaxel cytotoxicity.
Our results also suggest that a cyclin A–associated kinase other than CDK2 allowed cancer cells to progress from G2 to M, with increased cyclin B–associated kinase (CDK1) activity. One candidate for this cyclin A–associated kinase is CDK1 itself. Cyclin A is known to bind CDK1 and the resulting complex participates in the G2-M transition. However, we found here that overexpression of WT-CDK2, which can sequester cyclin A from cyclin A/CDK1 complexes (Fig. 4B), did not decrease sensitivity to paclitaxel (Fig. 3B). On the other hand, expression of DN-CDK2, which can also sequester cyclin A from cyclin A/CDK1 complexes to the same extent as WT-CDK2 (Fig. 4B), decreased sensitivity to paclitaxel (Fig. 1E).

Therefore, some kinase other than CDK1 is being complexed to cyclin A and activating CDK1, which is needed for paclitaxel sensitivity via its activation of spindle assembly checkpoint (24). Analysis of cyclin A level and formation of complexes in cell lysates immunodepleted of CDK2 might provide more insight into this issue.

Inhibitors of CDks, including CDK2, have been under development as cancer-therapy targets for more than a decade (9, 10, 46–49); one such agent, flavopiridol, has been tested in several clinical trials (50, 51) but has shown mixed results when combined with paclitaxel (52–56). Another line of evidence suggests that ectopic expression of CDK inhibitors can delay cell cycle progression and thus confer resistance to antitumor drugs that are cell cycle–dependent (e.g., 5-fluorouracil and doxorubicin; refs. 57, 58). Thus, it is possible that the less-than-optimal clinical results obtained when CDK inhibitors are given with chemotherapeutic drugs reflect the ability of some tumor cells to undergo cell cycle arrest rather than apoptosis. These clinical findings might also reflect the tumor cells having lost cyclin A–associated kinase and thus having become less sensitive to paclitaxel.

Other notable findings from our study are that expression of DN-CDK2 resulted in loss of Thr161-phosphorylated CDK1, which directly affects the loss of CDK1 activity, and that coexpression of cyclin A, but not CDK2, restored the phosphorylated CDK1 (Fig. 5). The phosphorylation of Thr161 is catalyzed by the cyclin H/CDK7 complex and is indispensable for induction of CDK1 kinase activity (36). The nature of a possible link between cyclin A–associated kinase or cyclin A and CDK-activating kinases, such as the cyclin H/CDK7 complex, remains to be elucidated.

Also of note was our deduction that expression of DN-CDK2 was not associated with accumulation of Tyr15-phosphorylated CDK1, which is suggested from the finding that the upper band of CDK1 disappeared when DN-CDK2 was expressed (Fig. 5). These findings contrast with those of another study of U2-OS osteosarcoma cells (59), which showed accumulation of Tyr15-phosphorylated CDK1 and loss of cyclin B protein level at S and G2 phase. Our findings agree with a report that incomplete DNA replication can prevent entry into mitotic phase independently of the inhibitory phosphorylation of CDK1 (60). They also agree with a report by Ducruet et al. (61) that DN-CDK2 expression (with or without coexpression of WT-CDK2) led to the induction of CDC25A phosphatase (data not shown). However, exactly how DN-CDK2 induces CDC25A and whether those two molecules directly affect the loss of CDK1 activity remain unclear. Therefore, the putative link between CDK2 inhibition and the loss of CDK1 activation seems to be more complicated than expected.

Figure 5. Changes in CDK1, phosphorylated CDK1, and cyclin B proteins by coexpression of WT-CDK2 or cyclin A with DN-CDK2. MDA-MB-468 cells were infected with the indicated combinations of Ad.mock, Ad.DN-CDK2, Ad.WT-CDK2, and Ad.cyclin A. At 24 h after infection, levels of CDK1, Thr161 (T161)–phosphorylated CDK1, cyclin B, and β-actin proteins were measured.

<table>
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Figure 4. Change in CDK1 activity by coexpression of WT-CDK2 or cyclin A with DN-CDK2. A, MDA-MB-468 cells were infected with the indicated combinations of Ad.mock, Ad.DN-CDK2, Ad.WT-CDK2, and Ad.cyclin A. At 24 h after infection, the cells were cultured in the presence or absence of paclitaxel for an additional 24 h. CDK1 (cyclin B–associated kinase) activity and immunoprecipitated cyclin B protein levels were measured. The ratio refers to the CDK1 kinase activity compared with that of the mock-infected cells. B, MDA-MB-468 cells were infected with the indicated combinations and MOI of Ad.mock, Ad.WT-CDK2, and Ad.DN-CDK2. At 24 h after infection, cyclin A level bound to immunoprecipitated cyclin B protein levels were measured. The ratio refers to cyclin A protein level compared with that of the mock-infected cells.
In conclusion, our findings suggest that cyclin A–associated kinase is the most important kinase for "pre-conditioning" cells to undergo paclitaxel-induced cell death. CDK2 activity is important to regulate the transition of cells up to pre-M phase, but it is not directly involved in regulating the CDK1, the activity of which is required for sensitivity to paclitaxel. This finding provides molecular confirmation that cells that do not enter mitosis will not be efficiently killed by paclitaxel. We also found that cyclin A–associated kinase is needed for entry into mitosis in addition to the requirement that CDK1 be activated for the spindle assembly checkpoint to be functional—another prerequisite for paclitaxel sensitivity (24). Thus, measurement of the cyclin A–associated kinase activity of tumors may prove to be a novel predictive marker of paclitaxel sensitivity. Finally, one may speculate that the addition of pure CDK2 inhibitors to taxanes for cancer chemotherapy may not modulate tumor cell sensitivity to taxanes but rather may increase the resistance of at least some of those cells to the taxanes.

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