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Inhibition of Cyclin-Dependent Kinase 2 by the Chk1-Cdc25A Pathway during the S-Phase Checkpoint Activated by Fludarabine: Dysregulation by 7-Hydroxystaurosporine

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ABSTRACT

Human myeloid leukemia ML-1 cells responded to cytostatic concentrations of fludarabine nucleoside (F-ara-A) by instituting an arrest in S-phase that involved the inhibition of cyclin-dependent kinase 2 (Cdk2). This seemed to be mediated by 1) persistent phosphorylation on the Tyr^{15} residue of Cdk2 and 2) an increased association of Cdk2 with p21. S-phase arrest was also associated with an increase in Chk1 kinase activity. Concomitantly, the activity of Cdc25A phosphatase was decreased. Immunoprecipitation studies demonstrated complexes of Cdk2, Cdc25A, and Chk1. The addition of the Chk1 kinase inhibitor 7-hydroxystaurosporine (UCN-01) to F-ara-A–arrested S-phase cells resulted in a rapid decrease in the fraction of cells with an S-phase DNA content and a corresponding increase in the fraction of apoptotic cells. Under these conditions, the kinase activity of Chk1 was reduced, Cdc25A phosphatase activity was increased, the level of Tyr^{15} phosphorylation of Cdk2 was reduced, and the kinase activity associated with immunoprecipitates of Cdk2 and cyclin A was reactivated. UCN-01 also had no effect on the association of p21 with Cdk2. Lastly, cells incubated with UCN-01 before F-ara-A addition did not arrest in S-phase. Thus, the DNA damage induced by F-ara-A initiated a hierarchical regulatory cascade through Chk1 and Cdc25A that resulted in Cdk2 inhibition, affecting an S-phase checkpoint that was dysregulated by UCN-01. These results suggest a mechanism by which UCN-01 enhances the cytotoxicity of agents that cause an S-phase arrest.

DNA damage inhibits critical cell cycle events controlled by checkpoint pathways (Mailand et al., 2000). After phosphorylation to the triphosphate, the nucleoside of fludarabine, 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), competes with dATP for misincorporation into DNA. This in turn blocks DNA replication by terminating the DNA strands and is quantitatively related to cytotoxicity (Huang et al., 1990; Sampath and Plunkett, 2000). Thus, F-ara-A is an S-phase–specific DNA-damaging agent. In ML-1 myeloid leukemia cells, the initial cellular responses are determined by the amount of drug incorporated: levels greater than 10^5 molecules per genome induce apoptosis (Sampath and Plunkett, 2000), whereas levels less than that cause arrest in the S-phase (Shi et al., 2001). Thus, analogous to checkpoint responses that potentially allow for the repair of DNA damage in the G_1 and G_2 phases (Poon et al., 1996; Sanchez et al., 1997; Liu et al., 2000; Mailand et al., 2000), cells may initiate a delay in cell cycle progression that stops DNA synthesis, thereby limiting incorporation of the analog. This may be a defense mechanism that circumvents toxicity.

Transit across S-phase is enabled by cyclin-dependent kinase 2 (Cdk2), a molecule that is regulated at multiple levels under normal conditions as well as during DNA damage. In response to DNA damage, cell-cycle arrest and inhibition of Cdk2 are associated with a persistent inhibitory phosphorylation on Tyr^{15} and linked to an inhibition of the Cdc25A phosphatase that dephosphorylates and activates Cdk2 (Mailand et al., 2000). Chk1 is a serine/threonine kinase that plays an important role as a DNA-damage induced checkpoint regulator by enforcing cell cycle arrest (Sanchez et al., 1997; Mailand et al., 2000; Cliby et al., 2002; Kohn et al., 2002). For instance, after ionizing radiation or replication blocks leading to G_2 arrest, the UV-induced G_1 checkpoint or topoisomerase inhibitor-induced S-phase delay, Chk1 inhibited the phosphatases Cdc25A (Mailand et al., 2000), Cdc25B, and Cdc25C (Peng et al., 1997; Sanchez et al., 1997; Kohn et al., 2002). However, a role for this cell cycle checkpoint regulator has not been established in the nucleoside analog-induced S-phase arrest.

Cdk2 is also regulated by binding to the CDK inhibitors

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ABBREVIATIONS: ara-C, 1-β-D-arabinofuranosylcytosine; Cdk, cyclin dependent kinase 2; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; UCN-01, 7-hydroxystaurosporine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

p21 and p27 (Toyoshima and Hunter, 1994; Harper et al., 1995; Ogryzko et al., 1997; Hengst et al., 1998). For example, in response to UV, ionizing radiation, or methylmethanesulfonate-mediated DNA damage, p21 is induced (Poon et al., 1996; Yuan et al., 1996) and attenuates Cdk2 activity, thus inhibiting progression into S-phase (Yuan et al., 1996; Brugarolas et al., 1999). Increased p21 activity is also associated with the down-regulation of Cdk2 in response to cisplatin-induced S-phase arrest (Knudsen et al., 2000). Furthermore, induction of p21 and inhibition of Cdk2 kinase are part of the apoptotic response to the related nucleoside analog ara-C (Yuan et al., 1996). In addition to directly inhibiting Cdk2 kinase activity, both p21 and p27 block the activating phosphorylation on Thr^{160} of Cdk2 by the CDK-activating kinase (Aprelikova et al., 1995).

UCN-01 is currently being evaluated in clinical trials both as a single agent and in combinations with fludarabine, ara-C, cisplatin, and 5-fluorouracil (Senderowicz, 2001). Clinically relevant amounts of UCN-01 that alone were innocuous to cells in culture enhanced the cytotoxicity of some chemotherapeutic DNA-damaging agents in vitro and in preclinical model systems (Akinaga et al., 1993; Wang et al., 1996; Shao et al., 1997; Sugiyama et al., 2000), thus providing a rationale for the use of UCN-01 in combination strategies for cancer treatment. A mechanism by which UCN-01 might enhance the cytotoxicity of DNA-damaging agents is its ability to abrogate cell cycle arrest after DNA damage (Bunch and Eastman, 1996; Lee et al., 1999; Graves et al., 2000; Jones et al., 2000; Sugiyama et al., 2000). To date, UCN-01 has been shown to abrogate cell cycle arrest and augment the toxicity of ionizing radiation, cisplatin, camptothecin, and mitomycin C (Bunch and Eastman, 1996; Shao et al., 1997; Graves et al., 2000; Sugiyama et al., 2000).

We have previously demonstrated the ability of nucleoside analogs such as ara-C, gemcitabine, and F-ara-A to induce S-phase arrest in human myelogenous leukemia cell lines. Furthermore, we have reported the actions of UCN-01 on cell cycle progression and viability of cells arrested by gemcitabine (Shi et al., 2001). In the present work, we investigated the effect of F-ara-A on the action of endogenous cell cycle checkpoint proteins that regulate S-phase to gain an understanding into the molecular mechanisms by which fraudulent nucleosides induce S-phase arrest. We also demonstrate a mechanism by which UCN-01 could abrogate the S-phase checkpoint response.

Materials and Methods

Cell Culture. ML-1, a human adult myelogenous leukemia cell line containing wild-type p53, was a gift from Dr. Michael B. Kastan (St. Jude Children's Research Hospital, Memphis, TN). Cells were maintained in exponential growth phase in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C (5% CO_2) in a humidified atmosphere. Population-doubling times were approximately 22 to 24 h.

Chemicals. F-ara-A was produced by the alkaline-phosphatase treatment of fludarabine (Berlex Laboratories, Richmond, CA). UCN-01 (NSC 638850) was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Aliquots of UCN-01 (10 mM in dimethyl sulfoxide) were stored at −20°C and diluted in RPMI immediately before each experiment. All other chemicals were reagent-grade.

Antibodies. Rabbit polyclonal antibodies to Cdk2 (sc-163), cyclin A (sc-751), 14–3-3σ (sc-629), Cdc25A (sc-7157), and Chk1 (sc-7898 and sc-8408) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to p21 (OP64 and OP68) were purchased from Oncogene Research Products (Cambridge, MA). Mouse monoclonal antibodies to cyclin E (14761) and p27 (13231A) were purchased from BD PharMingen International (San Diego, CA). Mouse monoclonal antibodies to Cdc25A, Cdc25B, and Cdc25C were purchased from Lab Vision (Fremont, CA). Rabbit polyclonal antibodies to Cdc25A (06-571) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody to Y^{15}-phospho-Cdk2 was purchased from Cell Signaling Technology (Beverly, MA). Histone H1 was purchased from Roche Applied Science (Indianapolis, IN).

TUNEL Assay. Cells were incubated with 1 μM F-ara-A for 24 h to induce S-phase arrest, and then UCN-01 was added to 50 nM to a portion of the culture for an additional 3 or 6 h. Cell pellets were washed with cold phosphate-buffered saline, fixed in 1% paraformaldehyde for 20 min on ice, and stored in 70% ethanol. The cells were analyzed for apoptosis using the APO-DIRECT kit for the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (BD PharMingen). Briefly, cells were prepared to simultaneously assess DNA nicks by TUNEL assay and DNA content by propidium iodide (PI) staining, which were analyzed simultaneously by flow cytometry using an Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA). The percentages of TUNEL-positive and -negative cells were obtained by standard analysis techniques using Elite software (Beckman Coulter). The cell cycle profiles were obtained using Multicycle software (Phoenix Flow Systems, San Diego, CA). The histograms were further divided into discrete analysis regions to quantitate the total percentage of cells as well as the percentages of TUNEL-positive and -negative cells that were in the G_1, S, and G_2/M phases of the cell cycle.

Cell Lysis. Briefly, 1–3 × 10^7 cells were pelleted by centrifugation at 1500*g* for 5 min, washed twice with ice-cold phosphate-buffered saline, and lysed on ice for 20 min in lysis buffer containing 25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.5% sodium deoxycholate, 20 mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, pH 8, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, pH 10, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Cells were centrifuged at 14,000*g* for 15 min at 4°C, and the supernatant was stored at −70°C until use. Protein content was determined using a protein assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Immunoprecipitation and Immunoblotting Analysis. For each sample, 500 μg of cell protein was mixed variously with 2 μg of anti-Cdk2, anti-cyclin A, anti-Cdc25A, or anti-Chk1 antibodies on a rocker for 3 h at 4°C. The immune complexes were then mixed with 100 μl of 20% protein A/G Sepharose beads (Oncogene Research Products) on a rocker for another 1 to 2 h at 4°C, and the beads were washed twice with lysis buffer. An equal volume of 2× SDS sample-loading buffer (100 mM Tris-Cl, pH 6.8, 20% glycerol, 4% SDS, 0.05% bromphenol blue, and 5% β-mercaptoethanol) was added to cells. Lysates were then heated at 95 to 100°C for 5 min. Aliquots (90 μg) of total cell protein were loaded onto 8 to 12% SDS-polyacrylamide gels (percentages depended on protein sizes detected), and proteins were electrophoresed at a constant voltage (70–100 V) and then electrotransferred to Immobilon-P membranes (Millipore, Bradford, MA) for 1 to 3 h at 250 mA. Membranes were blocked overnight in TBS-Tween 20 containing 5% nonfat dried milk and then incubated with primary antibodies (0.5–2 μg/ml) as indicated on the figures for 3 h and secondary antibodies conjugated to horseradish peroxidase (1:2500 dilution) for 1 h. The blots were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

Immunoprecipitation and Kinase Assay. For each sample, 500 μg of cell protein was mixed variously with 2 μg of anti-Cdk2, anti-cyclin A, anti-Cdk1, or anti-Chk1 on a rocker for 3 h at 4°C. The

immune complexes were then mixed with 100 μl of 20% protein A/G Sepharose beads (Oncogene Research Products) on a rocker for another 1 to 2 h at 4°C, and the beads were washed twice with lysis buffer, then once in kinase buffer (20 mM Tris-Cl, pH 7.5, 0.1 mM EGTA, pH 7.0, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The immunoprecipitates were incubated with 30 μl of kinase buffer plus 20 μM cold ATP, 1 μg/sample of histone H1 for the Cdk2, Cdk1, and cyclin A kinase assays (Roche Applied Science) or 2 μM cold ATP, 3 μg/sample GST-Cdc25A for the Chk1 kinase assay, and 6 μCi of [γ-^{32}P]ATP for 30 min at 37°C. An equal volume of 2× SDS sample-loading buffer was added to terminate the reaction. The mixture was then boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel. Autoradiography was performed, and a Betascope 603-blot analyzer (Betagen, Waltham, MA) quantified radioactivity. These data were expressed as specific activity after being normalized for the percentages of cells in S-phase as well as the amounts of Cdk2 and cyclin A present in the Cdk2 and cyclin A immunoprecipitates, respectively.

Cdc25A Phosphatase Activity. Cdc25A, Cdc25B, and Cdc25C phosphatase activities was measured on the basis of the activation of Cdk2. ML-1 cells were treated with 1 μM F-ara-A for 24 h to induce the phosphorylation of Cdk2 on Tyr^{15}. Inactive phosphorylated Cdk2 was then immunoprecipitated with antibodies to Cdk2 (200 μg of cell lysate per reaction). Cdc25A, -B, and -C were immunoprecipitated in parallel from 500 μg of cell extracts treated as indicated. Extracts from exponentially growing cells that lacked Cdc25A in the immunoprecipitation step was included as an experimental control. The beads from the Cdk2 and Cdc25 immunoprecipitates were mixed, incubated in 50 μl of a phosphatase buffer (20 mM Tris-HCl, pH 8.3, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 5 mM dithiothreitol) at 30°C for 1 h, and the reaction was stopped by addition of kinase assay buffer, after which the Cdc25 phosphatase activity was determined as a function of the histone H1 kinase activity of the Cdk2 immunoprecipitates.

Enrichment of Cells in S-Phase. ML-1 cells were exposed to 1 μM aphidicolin for 24 h to synchronize them at the G_1/S boundary. They were then washed in fresh RPMI medium to remove any traces of aphidicolin and resuspended in fresh medium to allow resumption of the cell cycle. The S-phase enriched fraction control used in the Cdk2 and CyclinA kinase assays were obtained from cells harvested 4 h after aphidicolin wash-out when approximately 75% of the cells were in S-phase as determined by PI staining and subsequent analysis by flow cytometry.

Statistics. Statistical analyses were performed with Microsoft Excel (Microsoft Corp., Redmond WA) and p values were obtained by unpaired t tests.

Results

F-ara-A–Induced S-Phase Arrest in Exponentially Growing ML-1 Cells: Dysregulation of Cell Cycle Arrest by UCN-01 Results in Enhanced Killing of S-Phase Cells. The S-phase fraction constituted approximately 40% of an exponentially growing population of ML-1 cells (Fig. 1A, a). There was a low percentage of cells with a sub-G_1 DNA content (2.0 ± 0.8%). When the cultures were exposed to a cytostatic concentration of F-ara-A (1 μM) for 24, 27, or 30 h, the S-phase fraction increased to approximately 60% (Fig. 1A, b-d) whereas the sub-G_1 DNA percentages of cultures at these times were 10.5 ± 0.8, 13 ± 1.9, and 15.8 ± 3.6%, respectively. Prior studies have indicated that compounds such as UCN-01 that abrogate the G_2 arrest induced by ionizing radiation (Wang et al., 1996), cisplatin (Bunch and Eastman, 1996), or DNA topoisomerase I inhibitors (Shao et al., 1997), potentiate the cytotoxicity of these agents. To evaluate the sensitivity of cells arrested in S-phase by a 24-h incubation with 1 μM F-ara-A, we exposed the arrested cells to an otherwise innocuous concentration of UCN-01 (50 nM) for 3 or 6 h. This resulted in a rapid decrease in the S-phase fraction (Fig. 1A, e-f) to 36 ± 7.4 and 29.4 ± 3.8%, respectively, compared with cells continually exposed to F-ara-A alone (Fig. 1, A, b-d, and C, b). The cells exposed to UCN-01 for 3 and 6 h also showed an increase in their sub-G_1 DNA content, to 25 ± 1.3 and 34.2 ± 3.7%, suggesting that S-phase-arrested populations were sensitized to UCN-01.

The TUNEL assay has the advantage of identifying DNA fragments in cells that are actively undergoing apoptosis versus cellular debris, which may be stained by propidium

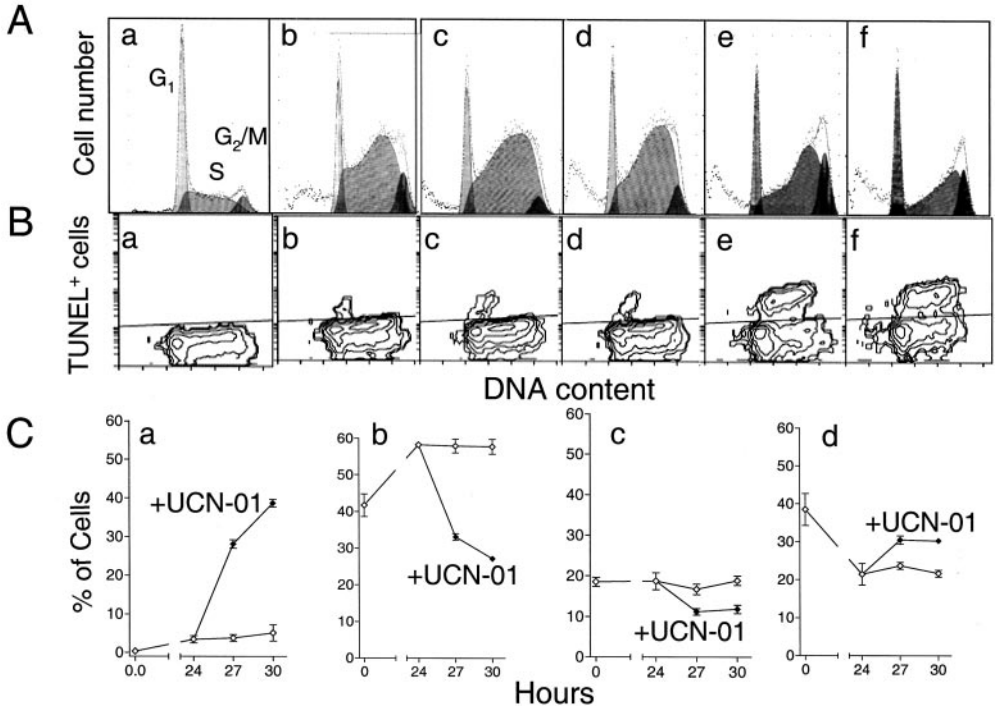

Fig. 1. F-ara-A–induced S-phase arrest in ML-1 cells: actions of UCN-01. Exponentially growing ML-1 cells were incubated with 1 mM F-ara-A for 30 h (◇), and 50 nM UCN-01 was added at 24 h to one portion (◆) when the culture was split. A, cells were fixed in 70% ethanol, stained with PI, and analyzed by flow cytometry to determine cell cycle progression. a, exponentially growing cells without F-ara-A; b to d, cells exposed to 1 μM F-ara-A for 24, 27, and 30 h, respectively; e and f, cells exposed to F-ara-A for 24 h, after which 50 nM UCN-01 was added for 3 or 6 h, respectively. B, cells from an experiment similar to the one above were harvested at the indicated times, fixed in 1% paraformaldehyde, and analyzed for apoptotic cells using the TUNEL assay. C, quantitation of the effect of F-ara-A and UCN-01 on the percentages of cells that were: TUNEL positive (a), in S-phase (b), in G_2-/M phase (c), and in G_1-phase (d).

iodide. The TUNEL assay was used to determine the percentages of apoptotic cells after exposure to either 1 μM F-ara-A alone for 24 to 30 h or this treatment for 24 h followed by incubation with 50 nM UCN-01 for 3 or 6 h. Exponentially growing ML-1 cells had low levels of DNA fragmentation (0.3% ± 0.1%) (Fig. 1, B, a, and C, a). Cells incubated with 1 μM F-ara-A for 24, 27, or 30 h demonstrated minimal increases in the TUNEL-positive fraction (3.4 ± 1% 3.8 ± 1, and 5.1 ± 2%, respectively) (Fig. 1, B, b-d, and C, a) in comparison with untreated ML-1 cultures. In contrast, cells incubated with F-ara-A and subsequently treated with UCN-01 for 3 or 6 h showed a steep increase in the fraction of TUNEL-positive cells to 28 ± 1 and 38 ± 1%, respectively (Fig. 1, B, e-f, and C, a), in comparison with cells treated with F-ara-A alone for the same amount of time. The data showed that the majority of TUNEL-positive cells seemed to arise from the portion of the population defined by PI staining to have an S-phase DNA content (Fig. 1B, e-f). This was accompanied by a reciprocal decrease in the TUNEL-negative population in the cells with an S-phase DNA content from approximately 60% in F-ara-A–arrested cells to 27% 6 h after the addition of UCN-01 (Fig. 1C, b). Furthermore, the addition of UCN-01 to F-ara-A–arrested cells did not result in an expansion of the G_2-/M population (Fig. 1C, c). On the contrary, there seemed to be a decrease in the G_2-/M population from approximately 18% of the whole population to about 11%, with a corresponding increase in the percentage of cells with a G_1 DNA content (Fig. 1C, c and d). Thus, the rapid loss of TUNEL-negative cells with an S-phase DNA content, the corresponding increase in apoptotic cells with an S-phase DNA content (Fig. 1, B and C, a-b), and the absence of an increase in the G_2-/M population (Fig. 1C, c) suggest that the addition of an otherwise nontoxic concentration of UCN-01 to S-phase-arrested cells initiated cell death without cell cycle progression into G_2-/M.

F-ara-A–Induced Inhibition of Cdk2 in S-Phase Cells. To evaluate the role of Cdk2 in F-ara-A–induced S-phase arrest, the activity of this kinase in cells exposed to F-ara-A was compared with that of active Cdk2 kinase in undamaged cells enriched for S-phase. S-phase-enriched populations, obtained as described under *Materials and Methods*, had approximately 75% of the total population in active S-phase as shown by PI staining (data not shown). Because the S-phase–enriched cells showed approximately the same percentage of the total population in S-phase as the F-ara-A–arrested cells, they served as an appropriate comparator against which to measure the Cdk2 activity of cells arrested in S-phase by F-ara-A. Using S-phase-enriched populations as a control also diminished the possibility of the Cdk2 kinase activity comprising of active cyclin E-Cdk2 as well as cyclin A-Cdk2 as seen in the Cdk2 immunoprecipitates from lysates of an exponential population. The specific activity of the kinase associated with immunoprecipitated Cdk2 in cells enriched for S-phase was 356 ± 4.5 cpm normalized to densitometry units of immunoblotted Cdk2 protein (Table 1). In contrast, cells arrested in S-phase by F-ara-A demonstrated a specific activity of only 199 ± 5.1 (P <0.01 for F-ara-A 24 h versus S-phase enriched fraction). During S-phase, the majority of the active Cdk2 is bound to cyclin A. Therefore, the cyclin A-associated histone H1 kinase activities were determined as a second measure of Cdk2 activity; S-phase–enriched populations had a specific activity of 352 ± 12.3 that decreased to 275 ± 6.4 in F-ara-A–arrested cells (P <0.05) (Table 1). Thus, F-ara-A treatment was associated with inhibition of Cdk2 kinase, which may represent the endpoint of the Chk1-Cdc25A checkpoint pathway that arrests cell cycle progression in S-phase after nucleoside analog-induced DNA damage.

TABLE 1
Specific activity of Cdk2 kinase.
Values are expressed as counts per minute normalized to Western blot densitometric units.

	Immunoprecipitated	
	Cdk 2	Cyclin A
S-enriched fraction	356 ± 4.5	352 ± 12.3
1 μM F-ara-A		
24 h	199 ± 5.1	275 ± 6.4
27 h	327 ± 7.8	272 ± 7.1
30 h	272 ± 7.6	315 ± 12.3
1 μM F-ara-A, 27 h		
+ 50 nM UCN-01, 3 h	381 ± 16.4	543 ± 10.1
1 μM F-ara-A, 30 h		
+ 50 nM UCN-01, 6 h	639 ± 2	697 ± 77

Addition of UCN-01 to F-ara-A–arrested cells for 3 and 6 h was associated with a reactivation in the kinase activity of Cdk2 to 381 ± 16 and 639 ± 2, respectively (P < 0.05 for F-ara-A 24, 27, or 30 h vs F-ara-A+UCN-01 3 or 6 h) (Table 1). There was a similar activation in cyclin A-associated kinase activity at these times to 543 ± 10 and 697 ± 77, respectively (P < = 0.01 for F-ara-A 24, 27, or 30 h versus F-ara-A+UCN-01 3 or 6 h) (Table 1). Thus, the UCN-01 seemed to activate Cdk2 in S-phase–arrested populations at the same time these cells initiated apoptosis (Fig. 1). This is consistent with other studies showing that the unscheduled activation of Cdk2 may facilitate apoptosis (Levkau et al., 1998). Lastly, the activity of immunoprecipitated Cdk1 did not increase in F-ara-A–arrested cells (specific activity = 154) in comparison with those exposed to F-ara-A and then subsequently to UCN-01 for 3 and 6 h (specific activities = 155 and 141, respectively). These results further support the conclusion that UCN-01 did not promote the progression of

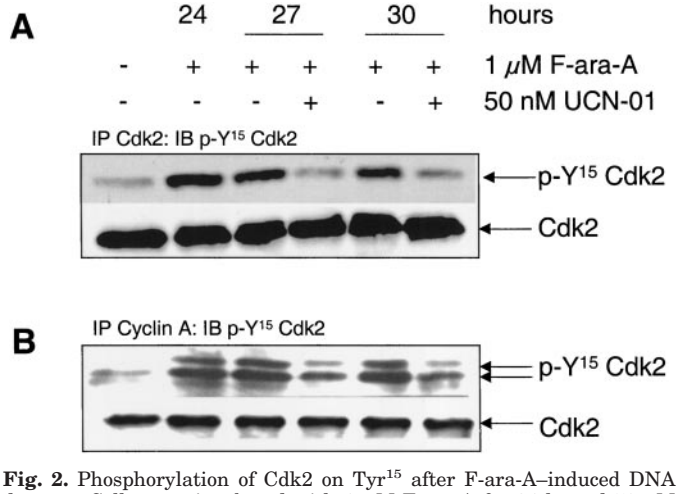

Fig. 2. Phosphorylation of Cdk2 on Tyr^{15} after F-ara-A–induced DNA damage. Cells were incubated with 1 μM F-ara-A for 24 h, and 50 nM UCN-01 was added at this time to one portion when the culture was split. Cells were harvested at the indicated times, and lysates were immunoprecipitated using antibodies to Cdk2 (A) or cyclin A (B). The upper blots demonstrate the levels of Tyr^{15} Cdk2 in the Cdk2 or cyclin A immunoprecipitates, whereas the lower blots indicate the amounts of Cdk2 present in the Cdk2 and cyclin A immunoprecipitates.

S-phase cells through the cell cycle; rather, it induced the selective killing of this S-phase-arrested population.

Cdk2 Is Phosphorylated on Tyr^{15} after F-ara-A–Induced DNA Damage. To determine whether the F-ara-A–induced inhibition of Cdk2 involved changes in the phosphorylation status of Tyr^{15} on Cdk2, extracts from exponentially growing ML-1 cells were immunoprecipitated with antibodies to Cdk2 as well as cyclin A. These were found to have low levels of Tyr^{15} phosphorylation on Cdk2 (Fig. 2). On exposure to F-ara-A for 24, 27, or 30 h, there was an increase in the levels of inhibitory Tyr^{15} phosphorylation in both the Cdk2 and cyclin A immunoprecipitates (Fig. 2). When F-ara-A–arrested cells were exposed to 50 nM UCN-01, there was an abrupt decline in the levels of Tyr^{15} phosphorylation in both the Cdk2 and cyclin A immunoprecipitates (Fig. 2), which correlated well with the increase in kinase activities observed in response to UCN-01 (Table 1).

Cdk2 Associates with Cdc25A. We then assessed whether endogenous Cdc25A phosphatase was directly associated with Cdk2. Lysates of cells were immunoprecipitated with anti-Cdc25A or anti-Cdk2 antibodies and probed for associated Cdk2 and Cdc25A, respectively. The results demonstrate an association between Cdk2 and Cdc25A phosphatase in exponentially growing cells that was not affected by F-ara-A–induced arrest or by subsequent treatment with UCN-01 (Fig 3A and B).

Cdc25A Is Inhibited in Response to F-ara-A–Induced DNA Damage. We next examined whether the accumulation in the Tyr^{15}-phosphorylated species of Cdk2 in response to F-ara-A was causally linked to an inhibition of Cdc25A activity. Exponentially growing cells were exposed to F-ara-A for 24 h, after which lysates were immunoprecipitated with antibodies to Cdc25A. The Cdc25A immunoprecipitates were coincubated with inactive Tyr^{15}-phosphorylated Cdk2, and the histone H1 kinase activity of the Cdk2 was assayed as a measure of the phosphatase activity of Cdc25A. As seen in Figure. 4A, arresting cells in S-phase with F-ara-A was associated with the inhibition of Cdc25A phosphatase. However, when F-ara-A–arrested cells were exposed to UCN-01 for 3 or 6 h, there was a reactivation in the phosphatase activity of Cdc25A (Fig. 4A, *upper blot*). Inhibition of Cdc25A has also been shown to result from the proteosome-mediated degradation of the protein after UV damage (Mailand et al., 2000). However, there was no decrease in the levels of immunoprecipitated Cdc25A protein in whole-cell lysates or in lysates of F-ara-A–arrested cells that were immunoprecipitated and then immunoblotted using antibodies against Cdc25A (see Figs. 3A and 5A, upper blots). In addition, no significant changes were detected in the association of 14–3-3 proteins with Cdc25A in F-ara-A–arrested cells or those cells subsequently exposed to UCN-01 compared with the levels found in exponentially growing cells (data not shown). Lastly, the activities of the related phosphatases Cdc25B and Cdc25C were unaffected by exposure to F-ara-A or UCN-01 (Fig. 4B). Taken together, our data indicate that the inhibition of Cdc25A phosphatase could form the basis of the F-ara-A–induced increase in the Tyr^{15} phosphorylation-associated inhibition of Cdk2 and institution of S-phase arrest. The reactivation of Cdc25A phosphatase by UCN-01 (Fig 4A) could explain the dephosphorylation of Tyr^{15} on Cdk2 and subsequent activation of Cdk2 kinase.

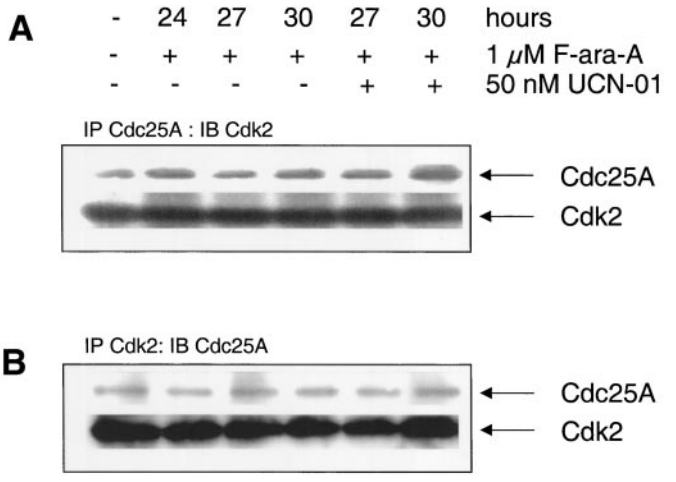

Fig. 3. Cdk2 associates with Cdc25A. Cells were treated as described in the legend to Fig. 2 and were then harvested as indicated. Lysates were immunoprecipitated using Cdc25A (A, upper blot) and immunoblotted to detect the associated Cdk2 (A, lower blot) or immunoprecipitated with Cdk2 (B, lower blot) and immunoblotted for associated Cdc25A (B, upper blot).

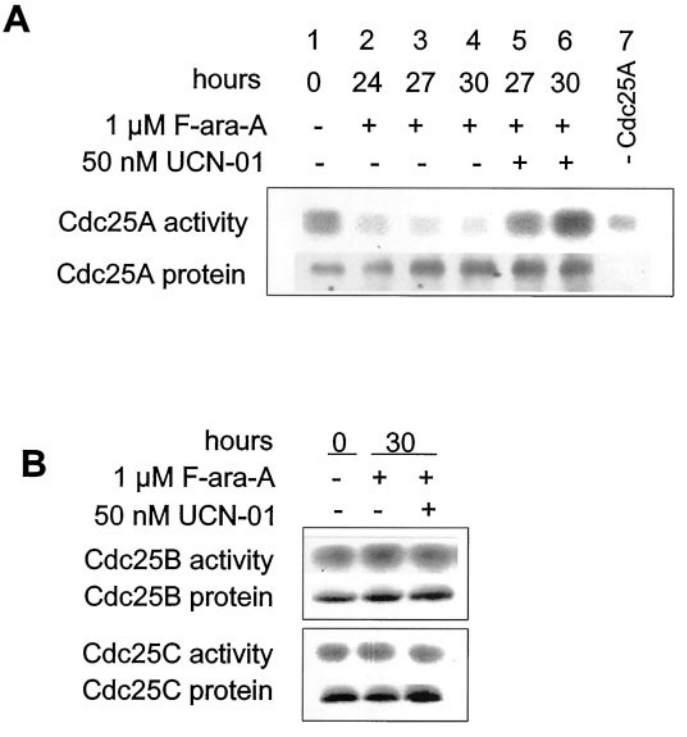

Fig. 4. Cdc25A phosphatase is inhibited by F-ara-A. Cells were treated as described in the legend to Fig. 2 and then harvested at the indicated times. A, Cdc25A phosphatase activity was measured in terms of the activation of Cdk2. Lysates were immunoprecipitated using antibodies to Cdc25A, except for lane 7, in which no Cdc25A immunoprecipitating antibody was added to a lysate from exponentially growing cells. Cdk2, which was phosphorylated on Tyr^{15}, was separately immunoprecipitated as described under *Materials and Methods*, and the beads from the Cdc25A and Cdk2 immunoprecipitates were mixed and coincubated for the phosphatase assay, after which Cdc25A phosphatase activity was measured in terms of the ability of the Cdk2 to phosphorylate histone H1 in a kinase assay (upper blot). The lower blot represents the amounts of immunoprecipitated Cdc25A in each sample. B, lysates were immunoprecipitated using antibodies to Cdc25B and Cdc25C and their phosphatase activities measured in terms of the activation of Cdk2 as described.

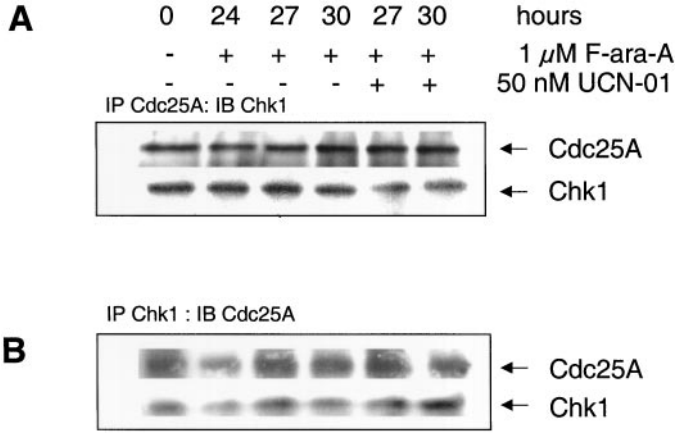

Fig. 5. Cdc25A associates with Chk1. Cells were treated as described in the legend to Fig. 2 and were then harvested as indicated. Lysates were immunoprecipitated using Cdc25A (A, upper blot) and immunoblotted for associated Chk1 (A, lower blot) or Chk1 (B, lower blot) and immunoblotted to detect the associated Cdc25A (B, upper blot).

Cdc25A Associates with the Checkpoint Kinase Chk1. The cell cycle checkpoint kinase Chk1 has been shown bind to and inhibit Cdc25A in vitro (Sanchez et al., 1997). To determine whether endogenously present Cdc25A phosphatase was directly associated with Chk1, lysates of cells were immunoprecipitated with Cdc25A antibodies and probed for associated Chk1 (Fig. 5A). Reciprocal immunoprecipitations using Chk1 antibodies that were immunoblotted to detect Cdc25A were also performed (Fig. 5B). The results demonstrate associations between Cdc25A and Chk1 in exponentially growing cells, F-ara-A–arrested S-phase cells and in cells subsequently exposed to UCN-01 (Fig. 5).

Chk1 Is Activated in Response to F-ara-A–Induced DNA Damage. The current concept of a cell cycle checkpoint response places the regulatory kinase Chk1 upstream from Cdc25A in the G_1-S checkpoint (Mailand et al., 2000). Chk1 also inhibits the activity of the related phosphatase Cdc25C in the maintenance of the DNA damage-induced G_2 checkpoint. We therefore determined whether this kinase was activated in response to the F-ara-A–induced intra-S-phase checkpoint. Exponentially growing cells, cells exposed to F-ara-A for 24, 27, or 30 h and cells exposed to F-ara-A and then

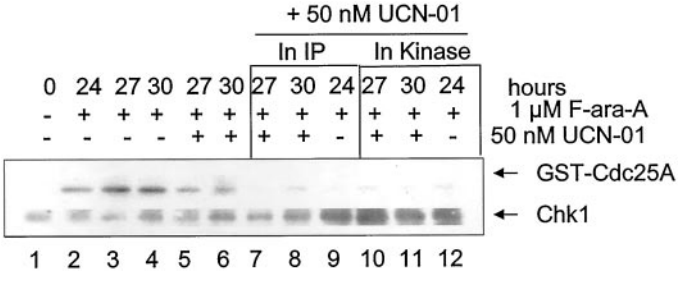

Fig. 6. F-ara-A activates Chk1. Cells were to 1 μM F-ara-A for 24 h, and 50 nM UCN-01 was added at this time to portions of the culture and then harvested at the indicated times. Lysates were immunoprecipitated with antibodies to Chk1, and their ability to phosphorylate GST-Cdc25A in an in vitro kinase assay was determined (upper blots, lanes 1–6). Lysates from cells exposed to 1 μM F-ara-A for 24 h or to F-ara-A and then UCN-01 for 3 or 6 h were also immunoprecipitated with Chk1 antibodies in the presence of 50 nM UCN-01 added either during the immunoprecipitation (upper blots, lanes 7–9) or kinase assay (upper blots, lanes 10–12). The lower blot indicates the amount of total Chk1 protein immunoprecipitated in the assay.

to 50 nM UCN-01 for 3 and 6 h were immunoprecipitated using antibodies to Chk1 and then assayed for their ability to phosphorylate exogenously provided GST-Cdc25A. Only cells exposed to F-ara-A demonstrated an increase in the kinase activity of Chk1 (Fig. 6, upper blot). When S-phase arrested cells were subsequently incubated with 50 nM UCN-01 there was a decrease in the kinase activity of Chk1, which is consistent with the role of UCN-01 as a Chk1 inhibitor (Fig. 6, upper blot, lanes 5 and 6). However, when lysates from these same cells were immunoprecipitated with Chk1 antibodies in the presence of 50 nM UCN-01 added either during the immunoprecipitation (Fig. 6, upper blots, lanes 7–9) or kinase assay (Fig. 6, upper blots, lanes 10–12), there was a loss in the kinase activity of Chk1. This suggests that UCN-01 directly inhibits the Chk1 kinase. Taken together, our results suggest that it is the UCN-01-induced loss in Chk1 kinase activity that results in the observed increases in Cdc25A phosphatase activity in S-phase arrested cells exposed to UCN-01 (Fig. 4A). Collectively, these data suggest that the F-ara-A–induced activation of Chk1 regulates Cdc25A and S-phase arrest, and that UCN-01, a potent inhibitor of Chk1, dysregulates this pathway.

UCN-01 Prevents F-ara-A–Induced S-Phase Arrest. On the basis of the data above, we investigated whether inhibition of Chk1 by exposing cells to UCN-01 before F-ara-A treatment would interfere with the ability of F-ara-A to induce an S-phase arrest. Exposure of exponentially growing cells to 1 μM F-ara-A for 24 h caused the expected increase in the S-phase population from 35% in control cells (Fig. 7a) to greater than 65% (Fig. 7b), which was reversed in cells subsequently exposed to 100 nM UCN-01 for 6-h (Fig. 7c). However, there was no change in the S-phase fraction in cultures treated with UCN-01 2 h before the addition of F-ara-A (Fig. 7d). Fig. 7e represents cells exposed to 100 nM UCN-01 alone. The failure of cells exposed concurrently to F-ara-A and UCN-01 to arrest in S-phase indicates that Chk1 function, maintenance of the Cdc25A phosphatase activity, and cyclin A-Cdk2 activity are critical regulators of S-phase arrest after F-ara-A–induced DNA damage.

Increased Recruitment of p21 But Not p27 into Cyclin A-Cdk2 Complexes After F-ara-A–Induced DNA Damage. A previous study has implicated p21 in mediating the down-regulation of Cdk2 kinase activity in response to cisplatin-induced S-phase arrest (Knudsen et al., 2000). Also, other studies have indicated the importance of p21, in addition to tyrosine phosphorylation, in the UV-induced inhibition of the related kinase Cdk4 (Terada et al., 1995). Therefore, the role of p21 in binding and inhibition of Cdk2 after F-ara-A–induced DNA damage was investigated. Cells were exposed to F-ara-A for 24 h, after which a portion of the cultures was exposed to UCN-01 for 3 and 6 h. Cell lysates were immunoprecipitated with antibodies to Cdk2 or cyclin A and assayed to determine the respective CDK partners as well as to measure the amounts of p21 associated with the cyclin A-Cdk2 complex. After exposure to F-ara-A alone or F-ara-A followed by UCN-01, the cyclin A-Cdk2 complex was intact in immunoprecipitates of Cdk2 and of cyclin A (Fig. 8, A and B, top and middle). Therefore, the inhibition of Cdk2 activity associated with F-ara-A treatment was not mediated by changes in the stability of the cyclin A associated with Cdk2. However, there was an increase in the amount of p21 associated with cyclin A or Cdk2 immunoprecipitates after

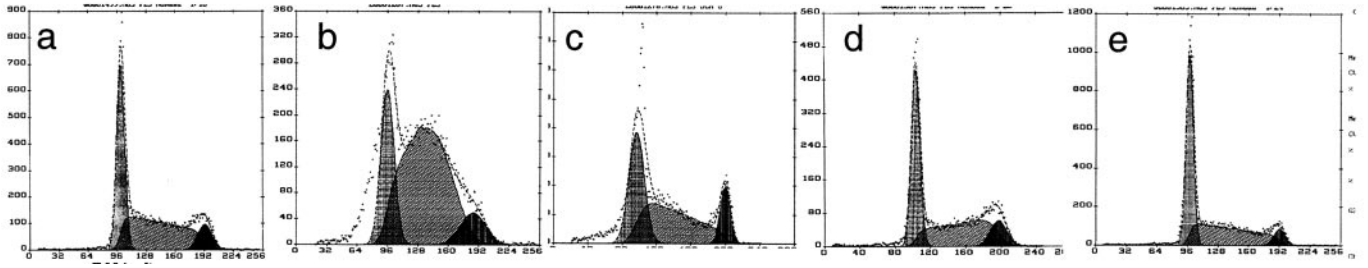

Fig. 7. Pretreatment with UCN-01 prevents F-ara-A induced S-phase arrest. a, untreated controls; b, cells exposed to 1 μM F-ara-A for 24 h; c, cells exposed to 1 μM F-ara-A for 24 h and then to 100 nM UCN-01 for 6 h; d, cells exposed to 100 nM UCN-01 for 2 h and then exposed to 1 μM F-ara-A for an additional 24 h; e, cells exposed to 100 nM UCN-01 alone (e). Cells were harvested, fixed in 70% ethanol, stained with PI, and analyzed by flow cytometry.

exposure of the cells to F-ara-A (Fig. 8, A and B, lower blots). Subsequent exposure of the S-phase–arrested cells to UCN-01 did not alter the levels of p21 in the cyclin A-Cdk2 complexes (Fig. 8, A and B, lower blots). In contrast, experiments using undamaged S-phase enriched populations demonstrated that little or no p21 was associated with Cdk2 in both the cyclin A and the Cdk2 immunoprecipitates (data not shown), indicating that p21 largely associated with Cdk2 in response to DNA damage. Thus, it is possible that the increased recruitment of p21 into cyclin A-Cdk2 complexes contributes to the inhibition of Cdk2 kinase after exposure to F-ara-A, and thus leads to S-phase arrest. Finally, because p27 has also been implicated in the regulation of Cdk2 kinase, we immunoprecipitated Cdk2 from exponentially growing cells, cells exposed to F-ara-A alone, and cells exposed to F-ara-A and then UCN-01. There was no significant change in the levels of p27 that were associated with Cdk2 before or after exposure to F-ara-A (Fig. 9) indicating that p27 was not involved in the F-ara-A-mediated inhibition of Cdk2.

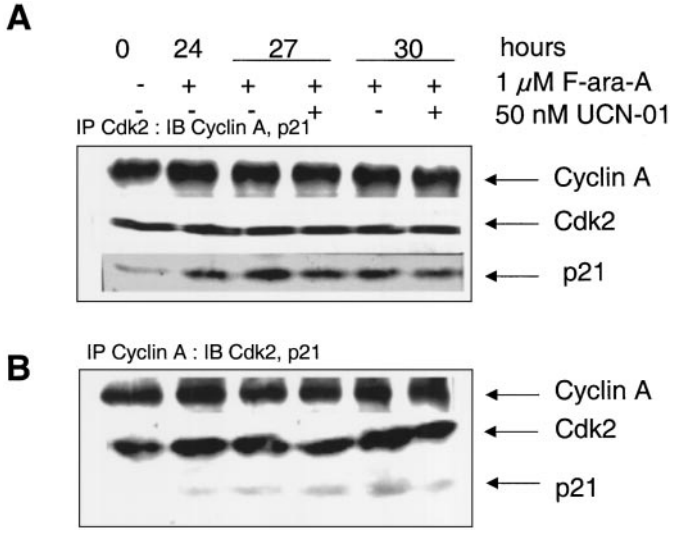

Fig. 8. Effect of treatment with F-ara-A at cytostatic concentrations on the association between p21 and Cdk2. Cells were treated as described in the legend to Figure. 2, and then harvested at the indicated times. A, lysates were immunoprecipitated using antibodies to Cdk2 and immunoblotted to detect associated cyclin A (upper blot) and p21 (lower blot) and the amounts of immunoprecipitated Cdk2 (middle blot). B, lysates were immunoprecipitated using antibodies to cyclin A and immunoblotted to detect associated Cdk2 (middle blot) and p21 (lower blot) and the amounts of immunoprecipitated cyclin A (upper blot).

Discussion

Cell-cycle checkpoints serve as surveillance systems to interrupt cell cycle progression when damage to the genome is detected. In the present study, we demonstrated that the DNA damage that occurs during replication as a result of the incorporation of nucleoside analogs such as F-ara-A activates an intra-S-phase checkpoint by signaling to effector molecules, thus causing a persistent S-phase arrest. We hypothesized that S-phase arrest represents a cellular defense mechanism that limits such genotoxic stress because disruption of F-ara-A–induced S-phase arrest by the addition of UCN-01 resulted in increased F-ara-A–induced cytotoxicity. The loss of S-phase cells induced by the action of UCN-01 on F-ara-A–arrested populations could reflect either an abrogation of S-phase arrest and progression into G_2-/M, or induction of apoptosis. Although UCN-01 has been shown to abrogate the S-phase arrest and enhance the toxicity induced by camptothecin in HT-29 colon carcinoma cells (Shao et al., 1997), our data showed that when the F-ara-A–induced S-phase arrest mechanism is dysregulated by the addition of UCN-01, the cells underwent apoptosis without progression into G_2/M.

Once incorporated into DNA, the F-ara-A nucleotide inhibits cellular DNA synthesis by its chain-terminating effect on nascent DNA (Huang et al., 1990). Although the mechanisms that detect DNA damage, which is likely to be manifested as stalled replication forks, and signal to arrest S-phase remain unknown, the proteins required for normal progression of the cell cycle, such as the cyclin-dependent kinases, are likely to

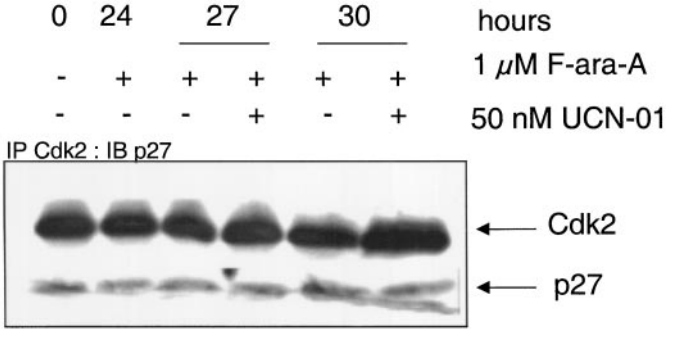

Fig. 9. Association of p27 with Cdk2 after exposure of cells to F-ara-A with or without UCN-01. Cells were treated as described in the legend to Figure 2, and then harvested at the indicated times. Lysates were prepared and immunoprecipitated with antibodies against Cdk2 Precipitates were immunoblotted to detect Cdk2 and associated p27.

be the critical targets of checkpoint signaling pathways that aim to halt cell cycle progression.

The DNA damage checkpoint kinase Chk1 plays a regulatory role upstream from Cdc25A and Cdk2 in the G_1-S checkpoint. Once activated, Chk1 can phosphorylate all three members of the Cdc25 family in vitro (Peng et al., 1997; Sanchez et al., 1997; Mailand et al., 2000). In the case of Cdc25A, the Chk1-mediated phosphorylation leads to inhibition of the phosphatase activity (Peng et al., 1997; Sanchez et al., 1997; Mailand et al., 2000). This prompted us to explore the possibility that Chk1 regulates the function of Cdc25A in the execution of the F-ara-A–induced S-phase checkpoint. We found that exposure of cells to F-ara-A induced the activity of Chk1 and corresponded closely with an inhibition in the Cdc25A phosphatase. However, when the F-ara-A–arrested cells were exposed to UCN-01, a known Chk1 inhibitor (Busby et al., 2000; Peng et al., 1997; Graves et al., 2000) there was a decrease in the kinase activity of Chk1, which was paralleled with an increase in the Cdc25A phosphatase and Cdk2 activity. This conclusion is supported by our data that demonstrated the inhibition of Chk1 kinase in the presence of UCN-01 during the immunoprecipitation or kinase assay (Fig. 6, lanes 7–12, upper blot)

Enforced expression of Cdc25A has been correlated with activation of cyclin E-Cdk2 and premature entry into S-phase (Mailand et al., 2000). We therefore hypothesized that inhibition of Cdc25A phosphatase could form the basis of the F-ara-A–induced inhibition of Cdk2 and establishment of the S-phase checkpoint. That this is the case is supported by our finding that F-ara-A–arrested cells exhibited an inhibition of the Cdc25A phosphatase that was paralleled by a persistent inhibitory phosphorylation on Tyr^{15} of Cdk2 (see Fig. 2) and corresponding inhibition of Cdk2 kinase activity. Exposure to UCN-01, led to an activation of Cdc25A that corresponded temporally with the activation of Cdk2 kinase and a rapid decrease in the levels of phospho-Tyr^{15} on Cdk2 (see Fig. 2), consistent with an abrogation of the S phase checkpoint response. As a corollary, the UCN-01–induced apoptosis of S-phase–arrested cells also seemed to involve an activation of Cdk2 kinase. Apoptosis elicited by a variety of agents is associated with the activation of cyclin A-associated kinases (Meikrantz et al., 1994). Conversely, dominant negative mutants of Cdk2 suppress apoptosis (Meikrantz and Schlegel, 1996; Levkau et al., 1998). Thus, the UCN-01–induced activation of endogenous Cdk2 kinase in F-ara-A–arrested cells may facilitate cell death.

Paradigms using the enforced over-expression of p21 to analyze the protein's functions have demonstrated that a single molecule of p21 is sufficient to inhibit the activity of Cdk2 (Hengst et al., 1998), and that p21 retards S-phase by inhibiting Cdk2 (Ogryzko et al., 1997). However, little is known about the role of p21 in inhibiting Cdk2 after the F-ara-A–induced activation of an S-phase checkpoint that arrests cell cycle progression in S-phase. p21, a critical regulator of Cdk2 function, was also induced after F-ara-A–mediated DNA damage. We found an increase in the recruitment of p21 into cyclin A-Cdk2 complexes after exposure to F-ara-A (see Fig. 8), suggesting that p21 contributes to the inhibition of Cdk2 and S-phase arrest. However, upon subsequent exposure to UCN-01, there was no significant loss of p21 from the cyclin A-Cdk2 complexes despite the observed increases in Cdk2 kinase activity. Therefore, it is likely that after F-ara-A exposure, p21 helps inhibit Cdk2 to arrest the cell cycle. However, exposure of arrested cells to UCN-01 probably activates signaling pathways other than those regulated by p21, such as those regulated by Chk1 and Cdc25A; this probably results in the unscheduled activation of Cdk2. Thus, we expect that in addition to p21, other cell cycle regulatory molecules cooperate to govern the action of Cdk2 in eliciting the F-ara-A–induced checkpoint response. That this is the case is supported by studies showing the need for the tyrosine phosphorylation of Cdk4, in addition to p21, in the G_1 arrest induced by UV radiation (Terada et al., 1995).

Finally, if the F-ara-A–induced S-phase checkpoint depended on the activities of Chk1, Cdc25A, and Cdk2, then based on the knowledge that UCN-01 was a Chk1 inhibitor, pre-exposing cells to UCN-01 would prevent the F-ara-A–induced activation of Chk1 and the resultant inhibition of Cdc25A phosphatase, thus maintaining Cdk2 activity. Our results demonstrated that pre-exposing cells for 2 h to UCN-01 before F-ara-A did indeed prevent the F-ara-A–induced S-phase arrest (Fig. 7). These results agree with another report that demonstrates diminished phosphorylation of Cdc25 phosphatase when UCN-01 was coadministered with F-ara-A (Harvey et al., 2001).

In summary, we investigated the mechanism by which F-ara-A activated an intra-S-phase checkpoint. We observed that there was an increased recruitment of p21, but not p27, into cyclin A-Cdk2 complexes after F-ara-A–induced S-phase arrest. The S-phase arrest was accompanied by a persistent phosphorylation on the Tyr^{15} residue of Cdk2 in response to F-ara-A; each of these events was associated with the inhibition of Cdk2 kinase. We demonstrated that the phosphorylation of Cdk2 is probably caused by the activity of a hierarchical regulatory pathway, initiated by a putative sensor of F-ara-A-terminated nascent DNA replication forks that subsequently activated Chk1 kinase, which in turn led to the inactivation of Cdc25A phosphatase. Signals through this pathway disrupted the normal equilibrium that curtails Tyr^{15} phosphorylation of Cdk2, culminating in increased phosphorylation and reduced Cdk2 kinase activity. When F-ara-A–arrested cells were exposed to the Chk1 kinase inhibitor UCN-01, there was a decrease in the kinase activity of Chk1. This was associated with reactivation of the Cdc25A phosphatase and dephosphorylation of the Tyr^{15} of Cdk2, thereby re-establishing its normal kinase function that allows cells to progress through S-phase. There was no evidence of such progression into G_2-/M, however, and cells seemed to initiate apoptosis within a few hours of the addition of UCN-01. We therefore conclude that, although the cells may be capable of resuming transit through the S phase, at least with regard to their Cdk2/cyclin A activity, this does not occur. Rather, S-phase-arrested cells are specifically targeted for death. Future investigations should evaluate the potential therapeutic advantage of strategies that dysregulate cell cycle checkpoints.

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