Inhibition of p34\textsuperscript{cdk2} Kinase Activity by Etoposide or Irradiation as a Mechanism of \textit{G}_2 Arrest in Chinese Hamster Ovary Cells\textsuperscript{1}

Richard B. Lock\textsuperscript{2} and Warren E. Ross\textsuperscript{3}

The J. Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky 40292

ABSTRACT

The mammalian homologue of the yeast cdc2 gene product, p34\textsuperscript{cdk2}, is a cell cycle-regulated protein essential for mitosis. We have used polyclonal antisera raised against a peptide corresponding to the carboxyl terminus of the sequence of human cdc2 to study p34\textsuperscript{cdk2} in Chinese hamster ovary (CHO) cells. Major bands are immunoprecipitated at a molecular weight of 34,000, although not in the presence of competing antigenic peptide. p34\textsuperscript{cdk2} coimmunoprecipitates with proteins of molecular weights of 52,000 and 57,000. Immunoprecipitates express histone H1 kinase activity which varies throughout the cell cycle, maximal activity being observed in G\textsubscript{2}-M. The activity of the p34\textsuperscript{cdk2} kinase varies according to its association with the M, 52,000 and 57,000 proteins and according to their phosphorylation state. Treatment of either asynchronous CHO cells or an enriched G\textsubscript{2} population with the antitumor agent, etoposide, results in rapid inhibition of immunoprecipitated p34\textsuperscript{cdk2} kinase activity, which is not due to a direct effect of drug upon the enzyme. p34\textsuperscript{cdk2} kinase activity recovers as cells arrest in G\textsubscript{2} and a second etoposide treatment further inhibits p34\textsuperscript{cdk2} kinase activity and prolongs G\textsubscript{2} arrest. Exposure of asynchronous CHO cells to \gamma-irradiation also inhibits p34\textsuperscript{cdk2} kinase activity within 1 h. Again this activity recovers as cells accumulate in G\textsubscript{2}. These results suggest that DNA damage in CHO cells elicits a response which results in inhibition of p34\textsuperscript{cdk2} kinase activity and, consequently, G\textsubscript{2} arrest.

INTRODUCTION

G\textsubscript{2} arrest is a response exhibited by proliferating eukaryotic cells exposed to a variety of DNA damaging agents including UV light (1), DNA alkylators (2), X-irradiation (3), radiomimetic agents such as bleomycin (4), and topoisomerase II inhibitors (5, 6). It is presumed that G\textsubscript{2} arrest allows cells to repair DNA lesions prior to mitosis. Caffeine exposure during G\textsubscript{2} induces mitosis before DNA repair is complete, resulting in enhanced cell killing (7). The biochemical mechanisms underlying G\textsubscript{2} arrest are not well understood. It has been proposed that G\textsubscript{2} arrest occurs as a result of the failure of essential genes to be transcribed, leading to an inability to divide (8, 9), although there is no direct evidence to support this hypothesis. Indeed, it seems equally likely that G\textsubscript{2} arrest results from a broad-based cellular response to DNA damage which alters the normal processes which regulate the transition of cells from G\textsubscript{2} to mitosis.

The antitumor epipodophyllotoxin, etoposide,\textsuperscript{4} appears to exert cytotoxicity via an interaction with the nuclear enzyme DNA topoisomerase II. Treatment of mammalian cells with this agent results in the rapid formation of DNA single- and double-strand breaks (10). These breaks involve the interaction of drug, DNA, and topoisomerase II to form a stable intermediate, termed the “cleavable complex” (11), in which one subunit of the topoisomerase II homodimer is covalently attached to the 5’-end of the broken DNA strand via a tyrosine residue (12). A linear correlation exists between etoposide-induced cytotoxicity and DNA double-strand breaks (13), although, upon removal of drug, rapid resealing of DNA lesions occurs (10). This indicates that cleavable complex formation induces a chain of events which results in cell death. Processes distal to cleavable complex formation are poorly understood, but have been proposed to involve illegitimate DNA recombination events (14) or an “SOS”-like response analogous to bacterial systems (15). As noted, one response which has been demonstrated for epipodophyllotoxins and all other topoisomerase II inhibitors is cell cycle arrest in the G\textsubscript{2} phase (5, 6).

The mechanisms which control cell cycle progression have been the subject of intensive research. Recently mammalian proteins have been identified which share homology with yeast cell division cycle control gene products, suggesting that elements of the mitotic control are conserved in all eukaryotes (16). Indeed, a homologue of the cdc2 gene product from \textit{Schizosaccharomyces pombe} is a component of maturation promoting factor (17), a cytoplasmic extract from metaphase-arrested \textit{Xenopus} oocytes that was originally characterized by its ability to induce meiosis in G\textsubscript{2}-arrested oocytes (18). The human homologue of the \textit{S. pombe} cdc2 gene product, p34\textsuperscript{cdk2}, is a protein kinase maximally active at the G\textsubscript{2}-M transition (19). Indeed, it copurifies with histone H1 kinase, predicting an intracellular function for this latter enzyme during mitosis (17). Enzymatic activity correlates with increased association of p34\textsuperscript{cdk2} with a M, 62,000 protein (p62, a human cyclin) and varies according to the phosphorylation state of p34\textsuperscript{cdk2} (19). The p34\textsuperscript{cdk2} kinase interacts directly with p13, the human homologue of the \textit{S. pombe} \textit{sic1} gene product (20), and with M, 50,000 to 60,000 proteins, cyclins A and B, in an active complex in the clam (21). Circumstantial evidence would imply that p34\textsuperscript{cdk2} plays a central role in the protein phosphorylation cascade which results in nuclear envelope disassembly, chromosome condensation, and construction of the mitotic spindle, by directly phosphorylating relevant proteins or stimulating other regulatory and effector enzymes (for review see Ref. 22). Microinjection of anti-p34\textsuperscript{cdk2} antibodies into serum-stimulated rat fibroblasts has yielded direct evidence that p34\textsuperscript{cdk2} is required in order for cell division to occur (23). Exit from mitosis is accompanied by inactivation of the p34\textsuperscript{cdk2}/cyclin complex, p34\textsuperscript{cdk2} phosphorylation, and cyclin proteolysis (21, 24).

The lack of understanding of G\textsubscript{2} arrest and cell death processes in mammalian cells led us to analyze the role that p34\textsuperscript{cdk2} may play in these important biological responses. We have used antisera prepared against the carboxyl terminus of human p34\textsuperscript{cdk2} protein to verify that p34\textsuperscript{cdk2} is a cell cycle-regulated protein in CHO cells, and that maximal activity is attained in G\textsubscript{2}-M. In addition, we demonstrate that treatment of CHO cells with either etoposide or \gamma-irradiation, at doses which result in G\textsubscript{2} arrest, causes rapid inhibition of p34\textsuperscript{cdk2} activity.
MATERIALS AND METHODS

Cell Culture, Synchronization, and Labeling. WT and etoposide-resistant (VpmR-5) CHO cell lines were maintained as monolayer cultures at 37°C in a-minimal essential medium supplemented with 5% FCS in a 5% CO₂ atmosphere. The selection and characterization of the resistant VpmR-5 subline have been described (25, 26). Drug treatments were carried out using 25 μM etoposide for 1 h at 37°C (1.5% survival compared with nondrug-treated controls by colony-forming assay) unless stated otherwise. A 10 mm working solution of etoposide was prepared immediately prior to use from a 100 mm stock dissolved in DMSO which had been stored at -20°C. Control cultures received the equivalent solvent exposure (0.04% for 25 μM etoposide). Following drug treatment, cells were rinsed twice with drug-free medium (37°C) and incubated in drug-free medium at 37°C. Cells were irradiated using a 60Co source at a dose rate of 196 cGy/min.

Synchronization of CHO cells was achieved using a double thymidine block (27). Exponentially growing cells were incubated at 37°C for 12 h in medium containing 2 mm thymidine, followed by a 6-h incubation in thymidine-free medium. Synchronization of cells at the G1-S boundary was then facilitated by a further 12-h incubation in thymidine-containing medium (2 mm).

For labeling of proteins, 1 to 2 x 10⁶ log-phase CHO cells were incubated for 2 h with 50 μCi/ml of Tran32P-label (ICN) in methionine-free medium, or with 100 μCi/ml of [35S]methionine (ICN) in phosphate-free medium.

Flow Cytometry. Approximately 10⁶ cells were harvested by trypsinization. After neutralization of the trypsin with medium containing 5% FCS, the cells were centrifuged (1000 rpm for 5 min), washed twice in ice-cold PBS, and finally resuspended in 0.3 ml of PBS. Cells were fixed by the gradual addition of 0.9 ml of ice-cold ethanol. After fixation, cells were washed twice with PBS and finally resuspended in 0.4 ml of PBS. Fifty μl of RNase A (5 mg/ml in PBS) and 50 μl of propidium iodide solution (50 μg/ml in 50 mM sodium citrate, pH 7.6) were then added. Cell cycle distribution was estimated using an Ortho Cytfluorograph 11s with an Ortho 2151 data handling system with doublet discrimination. The cell cycle distribution of ten thousand cells was analyzed and quantitated with reference to asynchronously dividing control samples.

p34cdc2 Kinase Studies. CHO cell extracts were prepared by lysis of monolayer cells in 50 mM Tris-Cl, pH 7.4, 250 mM sodium chloride, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM sodium fluoride containing the protease inhibitors antipain, aprotinin, chymostatin, leupeptin, and pepstatin A, each at a concentration of 20 μg/ml, and 2 mM PMSF. After incubation on ice for 30 min, the samples were centrifuged at 13,000 x g for 5 min at 4°C, and the supernatant was used for the subsequent assays. Aliquots were removed for protein determination (28) or quantification of TCA-precipitable radioactive material.

For immunoprecipitation reactions, equal amounts of protein (100 μg for kinase assays) or TCA-precipitable radioactivity (10⁻⁷ cpm for 35S- and 32P-labeling studies) were normalized by dilution with the above lysis buffer containing 1 mm PMSF. Immunoprecipitation was accomplished by the addition of 3 μl of affinity-purified polyclonal antiserum, raised against a synthetic peptide corresponding to the carboxy terminus of the human cdc2 protein (19); this was kindly provided by Dr. Giulio Draetta, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Titration of the antiserum revealed its optimal dilution to be 1:100 (data not shown). In some cases 100 nmol of competing antigenic peptide were included in the reactions. Following incubation for 1 h at 4°C, 3 mg of Protein A-Sepharose (Pharmacia) were added and the mixture was incubated on a rotator for 20 min at 4°C. Following centrifugation (13,000 x g for 10 s) pellets were washed 3 times with 1 ml of lysis buffer containing 1 mm PMSF at 4°C, and to the final pellet were added 60 μl of 2X SDS sample buffer [100 mM Tris-Cl (pH 6.8)-4% SDS-20 mM DTT-20% (v/v) glycerol-0.05% bromophenol blue], and the samples were boiled for 3 min and electrophoresed on a 7.5 to 4% SDS-20 mM DTT-20% (v/v) glycerol-0.05% bromophenol blue, and the radioactivity in each band was quantitated by excising the band and incubating it in 2 ml of 30% H₂O₂ overnight at room temperature. After the addition of 6 ml of ScintiVerse II (Fischer), radioactivity was determined by liquid scintillation counting.

Alkaline phosphatase treatments of immunoprecipitated proteins were carried out as previously described (19).

For Western blot analysis, proteins from cell lysates were electrophoresed as described above, transferred to nitrocellulose filters (29), and probed with anti-p34cdc2 antiserum. Bands were visualized using an alkaline phosphatase detection system (Bio-Rad Laboratories).

RESULTS

p34cdc2 Kinase Activity in CHO Cells. Immunoprecipitation of [35S]methionine-labeled CHO cell lysates, prepared from asynchronously dividing cells, with anti-cdc2 peptide serum followed by SDS-polyacrylamide gel electrophoresis resolved major bands with a molecular weight of approximately 34,000 (Fig. 1A, Lane 1), which were not precipitated in the presence of competing antigenic peptide (Lane 2). In addition, proteins with molecular weights of approximately 52,000, 57,000, and 64,000 were identified in immunoprecipitations carried out in the absence of competing antigenic peptide (compare Lanes 1 and 2 in Fig. 1A). HeLa p34cdc2 protein elutes at molecular weights of both 34,000 and >200,000 when subjected to gel filtration chromatography (19). The higher molecular weight complex includes a M₆, 62,000 protein, which has more recently been proposed to be a cyclin (21). Neither the M₆, 52,000 nor the 57,000 proteins were immunoprecipitated from CHO cells, which had been lysed in 50 mM Tris-Cl (pH 6.8)-0.5% SDS-1 labeled cell lysates as previously described (16) in a reaction volume of 25 μl containing 50 mM Tris-Cl, pH 7.4, 10 mm magnesium chloride, 1 mm DTT, 1 μM ATP, 50 μg/ml of histone H1 (Boehringer Mannheim), and 5 μCi of [γ-32P]ATP (specific activity, 3000 Ci/mmole). The radioactivity in each band was quantitated by excising the band and incubating it in 2 ml of 30% H₂O₂ overnight at room temperature. After the addition of 6 ml of ScintiVerse II (Fischer), radioactivity was determined by liquid scintillation counting.

Flow Cytometry. Approximately 10⁶ cells were harvested by trypsinization. After neutralization of the trypsin with medium containing 5% FCS, the cells were centrifuged (1000 rpm for 5 min), washed twice in ice-cold PBS, and finally resuspended in 0.3 ml of PBS. Cells were fixed by the gradual addition of 0.9 ml of ice-cold ethanol. After fixation, cells were washed twice with PBS and finally resuspended in 0.4 ml of PBS. Fifty μl of RNase A (5 mg/ml in PBS) and 50 μl of propidium iodide solution (50 μg/ml in 50 mM sodium citrate, pH 7.6) were then added. Cell cycle distribution was estimated using an Ortho Cytfluorograph 11s with an Ortho 2151 data handling system with doublet discrimination. The cell cycle distribution of ten thousand cells was analyzed and quantitated with reference to asynchronously dividing control samples.

p34cdc2 Kinase Studies. CHO cell extracts were prepared by lysis of monolayer cells in 50 mM Tris-Cl, pH 7.4, 250 mM sodium chloride, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM sodium fluoride containing the protease inhibitors antipain, aprotinin, chymostatin, leupeptin, and pepstatin A, each at a concentration of 20 μg/ml, and 2 mM PMSF. After incubation on ice for 30 min, the samples were centrifuged at 13,000 x g for 5 min at 4°C, and the supernatant was used for the subsequent assays. Aliquots were removed for protein determination (28) or quantification of TCA-precipitable radioactive material.

For immunoprecipitation reactions, equal amounts of protein (100 μg for kinase assays) or TCA-precipitable radioactivity (10⁻⁷ cpm for 35S- and 32P-labeling studies) were normalized by dilution with the above lysis buffer containing 1 mm PMSF. Immunoprecipitation was accomplished by the addition of 3 μl of affinity-purified polyclonal antiserum, raised against a synthetic peptide corresponding to the carboxyl terminus of the human cdc2 protein (19); this was kindly provided by Dr. Giulio Draetta, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Titration of the antiserum revealed its optimal dilution to be 1:100 (data not shown). In some cases 100 nmol of competing antigenic peptide were included in the reactions. Following incubation for 1 h at 4°C, 3 mg of Protein A-Sepharose (Pharmacia) were added and the mixture was incubated on a rotator for 20 min at 4°C. Following centrifugation (13,000 x g for 10 s) pellets were washed 3 times with 1 ml of lysis buffer containing 1 mm PMSF at 4°C, and to the final pellet were added 60 μl of 2X SDS sample buffer [100 mM Tris-Cl (pH 6.8)-4% SDS-20 mM DTT-20% (v/v) glycerol-0.05% bromophenol blue], and the samples were boiled for 3 min and electrophoresed on a 7.5 to 15% linear gradient SDS-polyacrylamide gel. For 3P-labeled proteins, gels were fixed with 7% acetic acid, dried, and autoradiographed. 35S-labeled proteins were detected using fluorography by impregnating the gel after fixation (7% acetic acid) with 1 m sodium salicylate, pH 6.8.

Kinase assays were carried out using immunoprecipitates from un-
mm DTT and boiled for 5 min (data not shown). This lysis procedure disrupts protein-protein complexes, suggesting that the M, 52,000 and 57,000 proteins exist in a high-molecular-weight complex with p34^cdc2_. Incubation of anti-cdc2 peptide immunoprecipitates with alkaline phosphatase resulted in the disappearance of the slower migrating bands with a molecular weight at around 34,000 (Fig. 1A, Lane 3). This treatment also resulted in less of the immunoprecipitated proteins being detected in the M, 40,000 to 90,000 range which, unlike the proteins in the M, 34,000 range, did not include a shift in mobility and may represent dilution of 35S-labeled protein. Immunoprecipitation of 32P-orthophosphate-labeled cell lysates with anti-peptide serum revealed a M, 34,000 doublet (Fig. 1A, Lane 4), which corresponded to the higher molecular weight forms from 35S-methionine-labeled lysates (compare Lanes 1 and 4).

Incubation of immunoprecipitates prepared from lysates of asynchronously dividing CHO cells with exogenously added histone H1 and [γ-32P]ATP resulted in phosphorylation of the histone (Fig. 1B, Lane 1). In the absence of exogenously added substrate a M, 57,000 protein was phosphorylated (Fig. 1B, Lane 2). No kinase activity was observed when the antecedent immunoprecipitation was carried out in the presence of competing antigenic peptide (Fig. 1B, Lane 3).

Thus, we have demonstrated that a protein with a molecular weight of approximately 34,000 (p34^cdc2_), which exists in phosphorylated and unphosphorylated forms, is specifically immunoprecipitated from CHO cells by affinity-purified human cdc2 antipeptide sera.

### p34^cdc2 Kinase Activity throughout the CHO Cell Cycle.

Using CHO cells which were synchronized at the G1-S boundary by a double thymidine block, we have evaluated the regulation of immunoprecipitated p34^cdc2 kinase activity throughout the cell cycle (Fig. 2A). In these experiments, p34^cdc2 kinase activity was immunoprecipitated from equal amounts of total cellular protein, exogenously added histone H1 was used as a substrate for phosphorylation. The kinase activity of immunoprecipitated p34^cdc2 increased as cells progressed through S phase (Fig. 2B, Lanes 1 to 3) and reached a peak in G2-M (Lanes 4 to 6). This activity decreased as cells divided and entered G1 (Fig. 2B, Lanes 7 and 8). It is unlikely that these changes were due to differences in cellular content of the p34^cdc2 protein, as Draetta and Beach (19) demonstrated that p34^cdc2 protein levels do not alter throughout the HeLa cell cycle. Furthermore, by Western blot analyses, we could detect no significant changes in the cellular content of p34^cdc2 throughout the CHO cell cycle (data not shown). Fig. 2C demonstrates that, as CHO cells progressed through S (Lanes 1 and 2) and into G2-M (Lanes 3 and 4), there was an increased association of p34^cdc2 with the M, 52,000 and M, 57,000 proteins in [35S]-methionine-labeled lysates, which decreased as cells entered G1 (Lane 5). In addition, the data indicate that p34^cdc2 and the M, 52,000 and 57,000 proteins existed in a higher phosphorylation state in G2-M cells compared with G1 cells (compare Lanes 10 and 11 in Fig. 2C). Thus, we have demonstrated that maximal immunoprecipitated p34^cdc2 kinase activity in CHO cells occurs during G2-M and that, during this period, p34^cdc2 is more highly phosphorylated and associated with M, 52,000 and 57,000 proteins.

#### Etoposide Treatment and p34^cdc2 Kinase Activity.

Treatment of CHO cells with etoposide (25 μM for 1 h) at 4 h postrelease of a double thymidine block, when over 50% of the cells were in G2-M (Fig. 2A; Fig. 3, Lane 1), caused significant inhibition of p34^cdc2 kinase activity (compare Lanes 2 and 8 in Fig. 3).

This activity did not recover until at least 8 h post-etoposide treatment (Lane 15). Lanes 6 and 7 in Fig. 3 represent normal turnover of p34^cdc2 activity as control cells progressed into G1.

Asynchronously dividing CHO cells which were exposed to etoposide (25 μM for 1 h) gradually accumulated in the G2-M phase of the cell cycle (approximately 80% at 12 h posttreat-
Histone HI occurred only in VpmR-5 cells at 50 μM etoposide (Fig. 6, Lanes 1 to 8). On the other hand, significant inhibition of p34<sub>cdc2</sub> activity was observed in WT cells (26). Purified topoisomerase II from the resistant cell line displays characteristics consistent with a mutant form of the enzyme (30). p34<sub>cdc2</sub> kinase activity was inhibited in WT cells at etoposide concentrations as low as 2.5 μM (Fig. 6, Lanes 1 to 8). On the other hand, significant inhibition of p34<sub>cdc2</sub> activity occurred only in VpmR-5 cells at 50 μM etoposide (Fig. 6, Lanes 9 to 16). Densitometric scans indicated that the etoposide concentrations required to induce equivalent inhibition of p34<sub>cdc2</sub> kinase activity in WT and VpmR-5 cells differed by a factor of ten. This is consistent with the degree of etoposide resistance exhibited by VpmR-5 cells. Thus, for etoposide-treated cells, DNA damage via cleavable complex formation appears a prerequisite for the cellular processes resulting in inhibition of p34<sub>cdc2</sub> kinase activity and G2 arrest.

The VpmR-5 cell line is 20- to 30-fold resistant to the cytotoxic effects of etoposide compared with WT cells (26). Purified topoisomerase II from the resistant cell line displays characteristics consistent with a mutant form of the enzyme (30). p34<sub>cdc2</sub> kinase activity was inhibited in WT cells at etoposide concentrations as low as 2.5 μM (Fig. 6, Lanes 1 to 8). On the other hand, significant inhibition of p34<sub>cdc2</sub> activity occurred only in VpmR-5 cells at 50 μM etoposide (Fig. 6, Lanes 9 to 16). Densitometric scans indicated that the etoposide concentrations required to induce equivalent inhibition of p34<sub>cdc2</sub> kinase activity in WT and VpmR-5 cells differed by a factor of ten. This is consistent with the degree of etoposide resistance exhibited by VpmR-5 cells. Thus, for etoposide-treated cells, DNA damage via cleavable complex formation appears a prerequisite for the cellular processes resulting in inhibition of p34<sub>cdc2</sub> kinase activity and G2 arrest.

The response of p34<sub>cdc2</sub> kinase to etoposide-induced cleavable complex formation suggests the possibility that other DNA-damaging agents which cause G2 arrest by treatment with etoposide. Asynchronously dividing cells were treated with etoposide (25 μM, 1 h), rinsed twice with drug-free medium (37°C), and reincubated in drug-free medium (C). At 10 h posttreatment cells were treated with etoposide in an identical manner as the first treatment and incubated in drug-free medium (D). p34<sub>cdc2</sub> kinase activity was assayed (Fig. 6).

The response of p34<sub>cdc2</sub> kinase to etoposide-induced cleavable complex formation suggests the possibility that other DNA-damaging agents which cause G2 arrest by treatment with etoposide. Asynchronously dividing cells were treated with etoposide (25 μM, 1 h), rinsed twice with drug-free medium (37°C), and reincubated in drug-free medium (C). At 10 h posttreatment cells were treated with etoposide in an identical manner as the first treatment and incubated in drug-free medium (D). p34<sub>cdc2</sub> kinase activity was assayed (Fig. 6).

The response of p34<sub>cdc2</sub> kinase to etoposide-induced cleavable complex formation suggests the possibility that other DNA-damaging agents which cause G2 arrest by treatment with etoposide. Asynchronously dividing cells were treated with etoposide (25 μM, 1 h), rinsed twice with drug-free medium (37°C), and reincubated in drug-free medium (C). At 10 h posttreatment cells were treated with etoposide in an identical manner as the first treatment and incubated in drug-free medium (D). p34<sub>cdc2</sub> kinase activity was assayed (Fig. 6).
Fig. 7. Inhibition of p34\textsuperscript{cdc2} kinase activity in CHO cells following γ-irradiation. Asynchronously dividing cells were irradiated with 800 cGy and harvested at 0 (Lane 1), 1 h (Lane 2), 2 h (Lane 3), 6 h (Lane 4), and 12 h (Lane 5) postirradiation. Lane 6 represents a mock-irradiated culture harvested at 12 h postirradiation. Cells were harvested, and p34\textsuperscript{cdc2} kinase activity was estimated.

DISCUSSION

Antiserum raised against a peptide corresponding to the carboxyl terminus of human cdc2 immunoprecipitates a M\textsubscript{r} 34,000 protein from CHO cell lysates. These immunoprecipitates express protein kinase activity in vitro in a cell cycle-regulated fashion. Kinase activity peaks in the G\textsubscript{2}-M phase, at which point the M\textsubscript{r} 34,000 protein exhibits maximal phosphorylation and coimmunoprecipitation with M\textsubscript{r} 52,000 and 57,000 proteins. Taken together, these data indicate that the M\textsubscript{r} 34,000 protein is the CHO homologue of the yeast cdc2 and human p34\textsuperscript{cdc2} proteins (16, 19).

Exposure of CHO cells to the topoisomerase II inhibitor, etoposide, at a concentration which induces G\textsubscript{2} arrest, results in a substantial and rapid inhibition of immunoprecipitated p34\textsuperscript{cdc2} kinase activity. This may be in response to cleavable complex formation rather than a direct effect of drug on the kinase, as treatment of a cell line which is etoposide resistant, by virtue of an altered topoisomerase II (30), does not result in inhibition of kinase activity. It is unlikely that VpmR-5 cells contain an altered p34\textsuperscript{cdc2} kinase in addition to an altered topoisomerase II, although the experiments described herein do not exclude this possibility. Irradiation of CHO cells also causes rapid inhibition of p34\textsuperscript{cdc2} activity, indicating that this response may be general to DNA-damaging agents. Currently, we are evaluating the effects of a broad range of antitumor agents on p34\textsuperscript{cdc2} kinase activity in CHO cells in order to determine whether this response is limited to those drugs which induce G\textsubscript{2} arrest, or whether it can be induced by any agent which interrupts cell cycle progression.

These observations raise interesting questions regarding the role played by p34\textsuperscript{cdc2} in the G\textsubscript{2} arrest response of eukaryotic cells. From this study it seems likely that inactivation of p34\textsuperscript{cdc2} kinase, a protein necessary for mitosis in mammalian cells (23), may participate in the processes leading to G\textsubscript{2} arrest. In our study, p34\textsuperscript{cdc2} activity is approximately 24-fold greater in G\textsubscript{2}-M than in G\textsubscript{1}, in agreement with other studies (19). Inhibition of p34\textsuperscript{cdc2} kinase activity in asynchronously dividing cultures, therefore, is due mainly to those cells which are in the G\textsubscript{2}-M compartment. At this juncture, however, it is not possible to determine whether identical responses to DNA damage are induced throughout the cell cycle. The precise mechanism of this observed inhibition of p34\textsuperscript{cdc2} activity is undefined. Cellular p34\textsuperscript{cdc2} content by Western blot, p34\textsuperscript{cdc2} synthesis, and the overall phosphorylation state estimated by [\textsuperscript{35}S]methionine and [\textsuperscript{32}P]orthophosphate incorporation studies, respectively, remain unchanged following etoposide treatment.\textsuperscript{8} The inactivation mechanism may involve turnover of cyclin-like proteins or discreet differences in phosphorylation of p34\textsuperscript{cdc2}, based on the known pathways of p34\textsuperscript{cdc2} regulation within the normal mammalian cell cycle (21, 24). However, any inhibitory mechanism proposed would have to be sufficiently short-lived in order to allow for the observation that p34\textsuperscript{cdc2} activity recovers as cells accumulate in G\textsubscript{2} (Fig. 4).

The gradual recovery of p34\textsuperscript{cdc2} activity in etoposide-treated or γ-irradiated CHO cells (Figs. 4 and 7) leads to the intriguing possibility that, in response to DNA damage, p34\textsuperscript{cdc2} activity is reset to an "early G\textsubscript{1}-like state." Interestingly, the time necessary for maximum recovery of p34\textsuperscript{cdc2} activity (between 10 and 18 h; Fig. 5) approximates the CHO population doubling time (around 15 h). Following recovery of p34\textsuperscript{cdc2} activity, G\textsubscript{2}-arrested cells are driven through mitosis, as shown by a decrease in G\textsubscript{2}-phase cells (Fig. 5B) and an increase in cell number (see Ref. 31). Retreatment of G\textsubscript{2}-arrested cells with etoposide leads to a further delay in G\textsubscript{2} by approximately 12 h, concomitant with inhibition and recovery of p34\textsuperscript{cdc2} activity (Fig. 5). Therefore, in the experiments described in this study, G\textsubscript{2} arrest may be explained by a rapid inhibition of an enzyme required for mitosis. Following recovery of p34\textsuperscript{cdc2} activity the enzyme may carry out the role required for mitosis.

Upon sustaining DNA damage, eukaryotic cells initiate a chain of events which results in G\textsubscript{2} arrest (4). This may allow repair of DNA lesions necessary for a successful mitosis. By artificially driving nitrogen mustard-treated baby hamster kidney cells through mitosis with caffeine, the drug's cytotoxicity is potentiated (7), indicating the protective role of G\textsubscript{2} arrest. It is prudent to observe that the mechanism by which caffeine interrupts G\textsubscript{2} arrest is unknown. In their study, Lau and Pardee (7) observed that, in cells induced to circumvent G\textsubscript{2} arrest, mitosis resulted in nuclear fragmentation. This phenomenon is also observed in etoposide-treated CHO cells which have overcome the G\textsubscript{2} blockade (see Ref. 31), indicating that agents which cause G\textsubscript{2} arrest via different types of DNA damage may induce similar pathways of cell death.

Until now, there has been little indication of the cellular events which may account for G\textsubscript{2} arrest in mammalian cells. Studies using radiation-sensitive yeast mutants indicate that the RAD9 gene product is necessary for G\textsubscript{2} arrest and protection from the cytotoxic effects of X-irradiation (1). The nature of this gene product is unknown, and an analogous mutation has yet to be isolated in mammalian systems. Rao (32) proposed that extensive chromosomal damage might account for irreversible G\textsubscript{2} arrest, and that specific cell cycle-regulated proteins necessary for mitosis were absent from G\textsubscript{2}-arrested cells. The latter explanation is also favored by Eastman's group when studying cisplatin-induced G\textsubscript{2} arrest (9). Specific transcripts or proteins absent in G\textsubscript{2}-arrested cells have yet to be identified. The data reported here suggest a different possibility, however. Specifically we hypothesize that the rapid loss of p34\textsuperscript{cdc2} kinase activity is one component of a general response of cells to DNA damage, perhaps in some way analogous to the bacterial "SOS" response (15).

An association between DNA damage, inhibition of p34\textsuperscript{cdc2} kinase activity, and G\textsubscript{2} arrest has been established. Much of the significance of this work is that it provides a pathway to examine the nature of the cellular response to cleavable complex formation. The mechanism of p34\textsuperscript{cdc2} kinase inhibition, the direct consequences of this inhibition on G\textsubscript{2} arrest, and the role of cell cycle-regulated proteins in the processes of cell death are now open to experimental characterization. In the accompa-
nying paper (31) we consider the potential relationship between G2 arrest and cell death.

ACKNOWLEDGMENTS

We gratefully acknowledge the helpful discussions with Dr. Giulio Draetta and Dr. Dan Sullivan and the Macintosh Computer for giving us the power to be our best!

REFERENCES

Inhibition of p34^cdc2 Kinase Activity by Etoposide or Irradiation as a Mechanism of G_2 Arrest in Chinese Hamster Ovary Cells

Richard B. Lock and Warren E. Ross


[Updated version](http://cancerres.aacrjournals.org/content/50/12/3761)