Identification of the functional domain of p21\textsuperscript{WAF1/CIP1} that protects cells from cisplatin cytotoxicity

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Yu, Fang, Judit Megyesi, Robert L. Safirstein, and Peter M. Price. Identification of the functional domain of p21\textsuperscript{WAF1/CIP1} that protects cells from cisplatin cytotoxicity. Am J Physiol Renal Physiol 289: F514–F520, 2005. First published April 19, 2005; doi:10.1152/ajprenal.00101.2005.—The p21 cyclin-dependent kinase (cdk) inhibitor protects cells from cisplatin cytotoxicity in vivo and in vitro. However, the mechanism of protection is not known. Separate p21 domains are known to interact with several different proteins having proapoptotic functions. To investigate the mechanism of protection by p21, we have constructed adenoviruses encoding the different domains of p21. We were able to localize the protective activity to a region of 54 amino acids containing the cyclin-cdk interacting moiety. Other protein binding domains of p21, including the NH\textsubscript{2}-terminal procaspase-3 interactive region and the COOH-terminal region containing the proliferating cell nuclear antigen binding domain and the nuclear localization signal, had little protective effect on cisplatin cytotoxicity. The dependence of cisplatin cytotoxicity on cdk2 activity was also demonstrated because 1) cisplatin caused a marked increase in cdk2 activity, which was prevented by the p21 expression adenovirus, and 2) a cdk2 dominant-negative adenovirus also protected cells from cisplatin-induced apoptosis. Thus the data suggest that the mechanism of p21 protection is by direct inhibition of cdk2 activity and that cisplatin-induced apoptosis is caused by a cdk2-dependent pathway.

CISPLATIN [cis-dichlorodiammine-platinum (II), CDDP] is widely used to treat several types of solid tumors in cancer patients (6). Its clinical utility is significantly limited by its nephrotoxicity, in which renal proximal tubule epithelial cells are especially sensitive (28). However, the mechanism by which cisplatin causes kidney cell death is unclear. Multiple mechanisms have been implicated in cisplatin-induced nephrotoxicity, which results in tubular cell death by apoptosis and necrosis (11, 25, 27, 30, 36, 40).

Murine models have been used to study cisplatin-induced acute renal failure (ARF). Mice injected with cisplatin undergo extensive renal cell death, and cultured proximal tubule cells undergo apoptosis after cisplatin exposure. Previous studies in our laboratory showed that p21, a cyclin-dependent kinase (cdk) inhibitor, was rapidly induced in the kidney in response to ARF produced by ischemia-reperfusion, ureteral obstruction, and cisplatin injection (21). The upregulation of the p21 gene ameliorated cisplatin-induced cell death in vivo and in vitro (18–20); p21\textsuperscript{-/-} mice showed increased cisplatin nephrotoxicity, and p21\textsuperscript{-/-} cells showed increased cisplatin cytotoxicity. Recently, by using a p21 expression adenovirus, and roscovitine, a cdk-inhibitory drug, we completely protected immortalized mouse kidney proximal tubule cells (TKPTS) (5) from cisplatin-induced apoptosis (29). The mechanism of protection by preexposure to p21 was independent of an effect on the cell cycle and was correlated with inhibition of caspase-3 activation.

p21 protein has separable domains that interact with proteins in different cellular compartments (3, 12, 16, 17, 24, 31, 33–35, 39, 41). The cyclin, cdk, and procaspase-3 binding domains are localized to the NH\textsubscript{2}-terminal of p21, whereas proliferating cell nuclear antigen (PCNA), c-Myc and calmodulin binding domains, as well as the nuclear localization sequence (NLS) are located in the COOH-terminal region.

To investigate the mechanism of p21 protection, we constructed adenoviruses encoding different domains of p21. We found that the NH\textsubscript{2}-terminal half of p21 protected TKPTS cells from cisplatin cytotoxicity with the same efficiency as full-length p21, whereas the COOH-terminal domains were not protective. Thus we separated the protective activity of the cdk-interacting moiety localized in the NH\textsubscript{2}-terminal region of p21 from the COOH-terminal PCNA-interacting moiety. This indicated that p21 did not require nuclear localization to intervene with the apoptosis pathway. To further localize the p21 protective domain, we constructed adenoviruses encoding amino acids (aa) 1–45 and 38–91 of p21 as green fluorescent protein (GFP) fusion proteins. We found that aa 38–91, containing the cdk2 binding domain, were protective, whereas the aa 1–45 fragment containing the procaspase-3-interacting moiety was not protective. Furthermore, cisplatin caused a marked increase in cdk2 activity that was prevented by adenovirus encoding protective p21 domains. The data demonstrated that the mechanism of the antiapoptosis effect of p21 was correlated with direct inhibition of cdk2 kinase activity and that cisplatin-induced apoptosis is caused by a cdk2-dependent pathway. This interpretation was further confirmed by a cdk2 dominant-negative adenovirus that also protected TKPTS cells from cisplatin-induced apoptosis.

MATERIALS AND METHODS

Reagents. Antibodies to p21, tubulin, and agarose-conjugated cdk2, as well as protein A/G plus agarose were obtained from Santa Cruz Biotechnology. cdk2 Monoclonal antibody and histone H1 were from Upstate Biotechnology. Antibodies to caspase-3 and cleaved poly (ADP) ribose polymerase (PARP) were from Cell Signaling Technology. [γ-\textsuperscript{32}P]ATP was obtained from Amersham Biosciences.

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**Cell culture and treatment.** Ad-293 cells (Stratagene) or HEK-293 cells (ATCC Cell Biology Collection) were grown at 37°C in 5% CO₂ in DMEM, supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum. Mouse kidney proximal tubule epithelial cells (TKPTS) were a gift from Dr. E. Bello-Reuss (5) and were maintained as described previously (29). Cisplatin was added to cultures to a final concentration of 25 μM when cells were ~75% confluent, and the cells were grown for an additional 24 h. Adenovirus was added to a final multiplicity of infection (MOI) of 100.

**Adenoviruses and adenoviral purification.** Human p21 cDNA (supplied by Dr. Bert Vogelstein, Johns Hopkins) was subcloned into pBluescript SK (+) plasmid (Stratagene). The p21 full-length and truncations (NH₂-terminal, COOH-terminal, aa 1–45 and aa 38–91) as GFP fusion proteins were generated by PCR amplification. The primers (Integrated DNA Technologies) used were the following: p21 full-length::GFP fusion protein, 5′-ACG AAA GCT TGG AGG CGC CAT GTG AGA ACC GGC GGC-3′, 5′-GAT GGA TCC GGG ATG GGT CAT CTG GGA GAT CAG-3′; NHOH-terminal::GFP fusion protein, 5′-ACG AAA GCT TGG AGG CGC CAT GTG AGA ACC GGC GGC-3′, 5′-GAT GGA TCC TCC TCA AAA ATG CTC ATC CCC CCG GCC GGC TCG-3′; COOH-terminal::GFP fusion protein, 5′-GTG CGC GAG GCC GGG AT, 5′-GAT GGA TCC CCT TCT GGA GAA GAT CAG-3′; aa 1–45::GFP fusion protein, 5′-ACG AAA GCT TGG AGG CGC CAT GTG AGA ACC GGC GGC-3′, 5′-GAT GGA TCC GGC GCT CCT GAT GTA GCA GCC GGC-3′; and aa 38–91::GFP fusion protein, 5′-ACG AAA GCT TAT AAT GGC CAT GTC AGA ACC GGC-3′; and aa 38–91::GFP fusion protein, 5′-GAT GGA TCC TCC TCC CAA CTA ATC CCC CCG GCC TCG-3′. The resultant plasmids were linearized by digestion with PmeI and subsequently cotransformed into Escherichia coli BJS183 cells (Stratagene) with pAdEasy-1 adenoviral backbone plasmid. Recombinants were selected for kanamycin resistance, and recombination using the AdEasy (8) vector system (supplied by Dr. B. Vogelstein). Fusion proteins using GFP and p21 full-length or truncations (NH₂-terminal, COOH-terminal, aa 1–45 and aa 38–91) were constructed using pEGFP-N3 plasmid (BD Biosciences Clontech) to fuse p21 in-frame with the NH₂-terminal end of GFP. The cassette with these genes was excised with HindIII and HpaI (New England Biolabs) and cloned into the HindIII/HpaI window of pAdTrack-CMV. The resultant plasmids were linearized by digestion with PmeI and subsequently cotransformed into Escherichia coli BJS183 cells (Stratagene) with pAdEasy-1 adenoviral backbone plasmid. Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analysis. Recombinant plasmids were linearized with PscI and transfected into Ad-293 cells. Recombinant adenovirus was amplified in HEK-293 cells, purified by CsCl banding, and stored at −20°C. Adenoviral infections were performed on TKPTS cells at 100 MOI (29).

**Western blot analysis.** Proteins were extracted from TKPTS cells using a lysis buffer containing 50 mM Tris·HCl, pH 7.4, 50 mM EDTA, 1% Triton X-100, supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals). Western blot analysis of each sample was performed using primary antibodies against p21 (BD Biosciences, 1:1000 dilution), p21 NLS (BD Biosciences, 1:1000 dilution), and p53 (BD Biosciences, 1:1000 dilution). Blots were developed using chemiluminescence reagents (Perkin Elmer Life Sciences). Blots were analyzed using ImageJ software (National Institutes of Health).

**Fig. 1.** Representation of various p21 constructions with different protein-interacting domains. Adenovirus encoding of full-length, NH₂-terminal (N-term), COOH-terminal (C-term), amino acids 1–45 (1–45aa) and amino acids 38–91 (38–91aa) of p21 as green fluorescent protein (GFP) fusion protein with or without different functional domains is shown. cd2k, cd2k Binding domain; PCNA, PCNA binding domain; procasp-3, procaspase-3 binding domain; NLS, nuclear localization sequence.

**Fig. 2.** Light microscopy of TKPTS cells before harvest. TKPTS cells were either untreated (A and C) or treated with 25 μM cisplatin (CP, B, D–H) in the absence (A) or presence of adenovirus expressing GFP (C) or p21 truncations (D–H) as GFP fusion proteins. A: cells were untreated. B: cells were treated with CP for 24 h. C: cells were treated with GFP adenovirus for 48 h. D: cells were treated with full-length::GFP adenovirus for 24 h and then for 24 h with CP. E: cells were treated with NH₂-terminal::GFP adenovirus for 24 h and then for 48 h with CP. F: cells were treated with NH₂-terminal::GFP adenovirus for 48 h and then for 24 h with CP. G: cells were treated with 1–45aa::GFP adenovirus for 24 h and then for 24 h with CP. H: cells were treated with 38–91aa::GFP adenovirus for 24 h and then for 24 h with CP. TKPTS cells were photographed using Hoffman optics before harvesting.
NaCl, 0.5% NP-40 with phosphatase and protease inhibitors (Sigma). Western blot analyses were performed as described previously (29). In brief, protein concentration was determined using a Bio-Rad protein assay. Protein (100 μg/lane) was electrophoresed using 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. After being blocked with 5% nonfat dry milk in TBST, the membrane was incubated at 4°C overnight with primary antibody. After washing, horseradish peroxidase-conjugated secondary antibody was applied. Proteins bound to the secondary antibody were visualized using enhanced chemiluminescence (Amersham Biosciences).

Immunoprecipitation and kinase assay. Proteins were prepared as above. The activity of cdk2 was determined by a modified histone H1 kinase assay (13). In brief, 200 μg of protein extract were immunoprecipitated by agarose-conjugated cdk2 antibody for 4 h at 4°C with constant rocking. After incubation, the agarose beads were washed with lysis buffer and kinase buffer. The pellets were then incubated in 20 μl of kinase buffer containing 2 μg histone H1, 20 μM ATP, and 10 μCi of [γ-32P]ATP for 30 min at 37°C. The reaction was stopped by Laemmli buffer (14). The samples were loaded on a 12% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film (Amersham Biosciences).

Light microscopy and immunofluorescence microscopy. Cells were photographed using Hoffman optics before harvesting (29). TKPTS cells infected with adenovirus encoding p21 NH2-terminal or COOH-terminal::GFP fusion proteins were grown on cover slides. Cells were fixed by 4% formaldehyde in PBS for 10 min and stained with DiIC16 (3) dye (Molecular Probes), a cell membrane-specific stain. Fluorescence images were taken using a fluorescence microscope with a Triple D-F-R (4′,6′-diamidino-2-phenylindole-FITC-rhodamine) filter and a ×40 objective.

Fluorescence-activated cell sorter analysis. The method used for fluorescence-activated cell sorter (FACS) analysis was described previously (29). Briefly, cells were harvested by trypsinization and collected by centrifugation. The cells were fixed in 70% ethanol at 4°C for 16 h. Cells were pelleted by centrifugation and resuspended in PBS-EDTA. The suspension was treated with RNase A and propidium iodide and analyzed using FACS Calibur. For each culture condition, ≥1 × 10⁴ cells were analyzed. The percentage of cells in sub-G₁/G₀ (apoptotic fraction) was determined using a cell cycle analysis program (WinMDI 2.8).

Statistical analyses. Statistical significance between control and treated groups was determined using Student’s t-test with two-tailed distribution.

RESULTS

Protective effect of NH2-terminal p21 on cisplatin-induced apoptosis in TKPTS cells. In the nucleus of most cells, p21 is constitutively present at low levels as a complex with cyclin-cdk and PCNA (3, 16, 17, 41). In addition to regulating cdk activity, p21 interacts with PCNA and interferes with its role in...
DNA replication (24, 39). The cdk and PCNA binding moieties of p21 are located separately (3, 17) and can be functionally separated by using truncated NH2-terminal or COOH-terminal domains (Fig. 1). To determine whether the protective domain of p21 can be restricted to these truncated proteins, TKPTS cells were infected with adenovirus encoding NH2-terminal, COOH-terminal, or p21 full-length::GFP fusion protein for 24 h. After cells were exposed to cisplatin (25 μM) for an additional 24 h, cell death was determined by light microscopy (Fig. 2), FACS analysis of propidium iodide-stained cells (Fig. 3, Table 1), and Western blot analysis for caspase-3 activation and PARP cleavage (Fig. 4).

Untreated TKPTS cultures had a background of 1.5% of cells as apoptotic and no detectable active caspase-3 or cleaved PARP. Treatment with cisplatin resulted in 25.6% of cells being apoptotic, with activation of caspase-3 and PARP cleavage. Transduction with full-length p21 or with the NH2-terminal half (aa 1–91) of p21 protected cells from cisplatin-induced apoptosis, which was lowered to 3.6 and 3.7%, respectively, and also prevented caspase-3 activation and PARP cleavage. The COOH-terminal half of p21 (aa 83–164) had little effect on cisplatin induction of apoptosis, which was 21.2%, or on caspase-3 activation and PARP cleavage. These p21 proteins were constructed as GFP fusions, and a negative control using a GFP expression adenovirus had no effect on the level of apoptosis.

In addition to cyclin and cdk binding domains, the NH2-terminal of p21 contains a procaspase-3 binding moiety at aa 1–33 (Fig. 1), which has been found to inhibit caspase-3 activation (33, 34). To determine whether the procaspase-3 binding domain contributes to the p21 protection effect, TKPTS cells were infected with adenovirus encoding aa 1–45 or 38–91 as GFP fusion protein and then treated with cisplatin as above. The aa 38–91 fragment was protective as confirmed by light microscopy (Fig. 2H) and FACS analysis (Fig. 3G, Table 1), whereas the aa 1–45 fragment was not protective (Figs. 2G and 3F, Table 1).

These data demonstrate that protection from cisplatin cytotoxicity by the intact p21 protein can be achieved by a p21 protein fragment only containing aa 38–91. At the same time, we cannot rule out the possibility that other domains of p21 that could contribute to protection when present in the intact protein might be functionally inactive in truncated molecules.

Binding of cdk2 to p21 truncations. The NH2-terminal region of p21 has been reported to contain the cdk binding domain between aa 49 and 71 (17, 32). We confirmed that protection by the NH2-terminal amino acids is associated with an interaction with cdk2. TKPTS cells were infected with adenovirus encoding NH2-terminal, COOH-terminal, or full-length p21::GFP fusion proteins and then exposed to cisplatin. Whole cell lysates were immunoprecipitated with antibody to human p21 followed by Western blotting with cdk2 antibody. We found that full-length and NH2-terminal p21 bound to cdk2 after cisplatin treatment, whereas the COOH terminus did not bind cdk2 (Fig. 5A). To determine whether the smaller truncations also bind cdk2 in vivo, TKPTS cells were infected with adenovirus encoding GFP, full-length p21, aa 1–45 fragment, or aa 38–91 fragment and then exposed to cisplatin. Available p21 antibodies do not recognize both of the smaller p21 fragments, so that immunoprecipitations were performed using anti-GFP to recognize the GFP moiety of the p21 fusion proteins, followed by Western blotting with cdk2 antibody. We found that full-length and NH2-terminal p21 bound to cdk2 after cisplatin treatment, whereas the COOH terminus did not bind cdk2 (Fig. 5B).

Subcellular localization of p21 fragments. To determine subcellular localization of truncated p21, the TKPTS cells were infected with adenovirus encoding NH2-terminal or COOH-terminal as GFP fusion protein, respectively. As shown in Fig. 6A, the NH2 terminus of p21 was localized primarily to the cytoplasm, whereas the COOH terminus containing NLS was

Table 1. Percentage of apoptotic (sub-G0/G1) cells after cisplatin treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of Cells</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5±0.5</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>25.6±2.7</td>
<td></td>
</tr>
<tr>
<td>CP + full-length</td>
<td>3.6±0.5</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>CP + N-term</td>
<td>3.7±0.8</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>CP + C-term</td>
<td>21.2±2.3</td>
<td>NS</td>
</tr>
<tr>
<td>CP + 1–45aa</td>
<td>23.4±1.9</td>
<td>NS</td>
</tr>
<tr>
<td>CP + 38–91aa</td>
<td>7.7±1.4</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>CP + dn-cdk2</td>
<td>1.3±0.6</td>
<td>P&lt;0.01</td>
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Results are means ± SD (n = 3). TKPTS cells were untreated (control), treated with 25 μM cisplatin for 24 h (CP), or treated with adenovirus encoding full-length (CP + full-length), NH2-terminal (CP + N-term), COOH-terminal (CP + C-term), 1–45aa fragment (CP + 1–45aa), or 38–91aa fragment (CP + 38–91aa) of p21 as GFP fusion protein, dominant-negative cdk2 (CP + dn-cdk2) for 24 h, and then for 24 h with CP. NS, not significant. Percentage of cells in the phase of sub-G0/G1 (apoptotic fraction) was determined by fluorescence-activated cell sorter analysis. P values are compared with the CP-treated group.
half of p21 inhibits cdk kinase activity very weakly (4). We collected using a fluorescence microscope of p21. TKPTS cells were treated with adenovirus encoding NH2-terminal and COOH-terminal of p21. TKPTS cells were infected with GFP fusion proteins for 24 h and then for 24 h with 25 μM CP. Whole cell lysates were immunoprecipitated (IP) by antibody to human p21 followed by Western blotting with cdk2 antibody.

**Cisplatin-induced apoptosis is associated with increased cdk2 activity.** The cdk2 protein level was not changed under cisplatin treatment compared with untreated TKPTS cells (data not shown). Whole cell lysates were immunoprecipitated by cdk2 antibody, and the cdk2 activity was determined by histone H1 kinase assay. TKPTS were treated with adenovirus encoding either full-length (CP + full length), NH2-terminal (CP + N-term), COOH-terminal (CP + C-term) of p21 as GFP fusion proteins, dominant-negative cdk2 (CP + dn-cdk2), or wild-type cdk2 (CP + wt-cdk2) for 24 h. Protein extract was immunoprecipitated by agarose-conjugated cdk2 followed by kinase assay with histone H1. Background was indicated by agarose-conjugated cdk2 antibody incubated with kinase buffer (background).

**DISCUSSION**

Cisplatin is an effective chemotherapeutic drug, and the major disadvantage of this antineoplastic agent is a dose-dependent and cumulative nephrotoxicity. The proximal tubule localized in the nucleus (Fig. 6B). These results demonstrate that the p21 did not require nuclear localization to intervene with the cell death pathway activated by cisplatin.

**Fig. 5.** Binding of cdk2 to p21 truncations. A: binding of cdk2 to NH2-terminal of p21. TKPTS cells were treated with adenovirus encoding NH2-terminal (CP + N-term), COOH-terminal (CP + C-term), or full-length (CP + full length) as GFP fusion protein for 24 h and then for 24 h with 25 μM CP. Whole cell lysates were immunoprecipitated (IP) by antibody to human p21 followed by Western blotting with cdk2 antibody. B: binding of 38–91aa fragment of p21 to cdk2. TKPTS cells were infected with GFP adenovirus for 48 h (GFP), or treated with adenovirus encoding 1–45aa fragment (CP +1–45aa), 38–91aa fragment (CP +38–91aa), or full-length p21 (CP + full length) as GFP fusion protein for 24 h and then for 24 h with CP. Proteins were immunoprecipitated by GFP antibody and then probed with primary antibodies either to cdk2 or GFP. Lysis buffer containing anti-GFP was used to indicate background (background). The p21::GFP fusion protein has a similar molecular weight to IgG heavy chain (53-kDa) as indicated in bottom panel.

**Fig. 6.** Subcellular localization of NH2-terminal and COOH-terminal of p21. TKPTS cells treated with adenovirus encoding NH2-terminal (A) or COOH-terminal (B) of p21 as GFP fusion protein were grown on coverslips. Cells were fixed and then stained with DiIC16 (3) dye. Fluorescence images were collected using a fluorescence microscope with a triple 4′,6′-diamidino-2-phenylindole–FITC–rhodamine filter and a ×40 objective. Both fields are the same magnification. Green stain, NH2-terminal::GFP or COOH-terminal::GFP fusion protein. Red stain, DiIC16 (3) fluorescent dye membrane stain.

**Fig. 7.** CP-induced increase in cdk2 activity is prevented by full-length and NH2-terminal domain of p21 and by dominant-negative cdk2. The activity of cdk2 was determined by histone H1 kinase assay. TKPTS were treated with GFP adenovirus alone (GFP) for 48 h, untreated (control), or treated with 25 μM CP (CP) for 24 h. Before treatment with CP, some cultures were treated with adenovirus encoding either full-length (CP + full length), NH2-terminal (CP + N-term), COOH-terminal (CP + C-term) of p21 as GFP fusion proteins, dominant-negative cdk2 (CP + dn-cdk2), or wild-type cdk2 (CP + wt-cdk2) for 24 h. Protein extract was immunoprecipitated by agarose-conjugated cdk2 followed by kinase assay with histone H1. Background was indicated by agarose-conjugated cdk2 antibody incubated with kinase buffer (background).

To confirm that cdk2 plays a key role in cisplatin-induced apoptosis, TKPTS cells were infected with adenovirus encoding dominant-negative cdk2 or wild-type cdk2 for 24 h followed by cisplatin exposure. Whole cell lysates were used for cdk2 kinase assay or Western blotting with antibodies to casepase-3, cleaved PARP, and tubulin. As shown in Fig. 7, cdk2 activity was inhibited completely by the dominant-negative cdk2 adenovirus, whereas wild-type cdk2 had no effect. Similar to p21, dominant-negative cdk2 also protected against cisplatin-induced apoptosis, whereas wild-type cdk2 did not (Fig. 4). Dominant-negative cdk2 also protected against apoptosis as measured by morphology (data not shown) or FACS analysis showed that cisplatin-induced apoptosis was reduced to the untreated level in which apoptosis was 1.3 ± 0.6% (Fig. 3H, Table 1).

**Fig. 7.** CP-induced increase in cdk2 activity is prevented by full-length and NH2-terminal domain of p21 and by dominant-negative cdk2. The activity of cdk2 was determined by histone H1 kinase assay. TKPTS were treated with GFP adenovirus alone (GFP) for 48 h, untreated (control), or treated with 25 μM CP (CP) for 24 h. Before treatment with CP, some cultures were treated with adenovirus encoding either full-length (CP + full length), NH2-terminal (CP + N-term), COOH-terminal (CP + C-term) of p21 as GFP fusion proteins, dominant-negative cdk2 (CP + dn-cdk2), or wild-type cdk2 (CP + wt-cdk2) for 24 h. Protein extract was immunoprecipitated by agarose-conjugated cdk2 followed by kinase assay with histone H1. Background was indicated by agarose-conjugated cdk2 antibody incubated with kinase buffer (background). The pellets were incubated in 20 μl of kinase buffer containing 2 μg histone H1, 20 μM ATP, and 10 μCi of [γ-32P]ATP at 37°C with 30 min. The reaction was stopped by Laemmli buffer. Samples were loaded on a 12% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film.
segment of the nephron is the most sensitive to cisplatin and undergoes severe damage in response to cisplatin-based chemotherapy (28). ARF after cisplatin administration is associated with increased cell cycle activity, in which many normally quiescent kidney cells enter the cell cycle. There are increased nuclear PCNA levels, as well as [3H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) incorporation into nuclear DNA (18, 20). It is clear that apoptosis is typically increased in proliferating cell populations. However, the mechanisms underlying the link between apoptosis and proliferation following injury are poorly defined. A coordinated activation of specific cdks by partner cyclins is necessary for proliferation, and several studies have associated cdk2 activity with apoptosis (1, 7, 9, 22, 23).

Coincident with increased cell cycle activity by cisplatin-induced ARF, we have shown a rapid induction of the p21 gene (21). Although originally described as a cdk inhibitor, p21 has also been shown to oppose apoptosis following a variety of apoptotic stimuli (2, 15, 33, 34). Our previous studies have demonstrated that induction of p21 after cisplatin administration has a protective effect on the murine kidney in vivo (20), on long-term cell cultures (19), and on primary renal proximal tubule cultures (26). These in vitro studies showed that the lack of p21 was associated with the acceleration of caspase activation and apoptosis. We have shown that preexposure to p21 adenovirus protected TKPTS cells from cisplatin-induced apoptosis and have speculated that cell cycle inhibition per se is not the sole mechanism of the protection (29).

Cytoplasmic localization of p21 appears to be critical in determining its antiapoptotic ability (2, 15). In this study, we found that p21 did not require nuclear localization to intervene with the cell death pathway initiated by cisplatin and that nuclear localization of a p21 fragment not containing the cdk2 binding domain was insufficient to protect. These data were consistent with previous studies that cyclin A-ckd2 and cyclin E-ckd2 complexes shuttle between the nucleus and cytoplasm (10) and that the translocation of nuclear cyclin A-ckd2 to the cytoplasm was associated with an increased kinase activity during apoptosis (9). Indeed, the cytoplasmic localization of cdk2 was correlated with apoptosis rather than proliferation of mouse kidney mesangial cells (9). Many cytoplasmic proteins have been demonstrated to be substrates for yeast cdk (37), and mouse kidney mesangial cells (9). Many cytoplasmic proteins have been demonstrated to be substrates for yeast cdk (37), and that the translocation of nuclear cyclin A-cdk2 to the cytoplasm was associated with an increased kinase activity during apoptosis (9). Indeed, the cytoplasmic localization of cdk2 was correlated with apoptosis rather than proliferation of mouse kidney mesangial cells (9).

In conclusion, our data show that the cdk2 binding domain of p21 is critical to determine its ability to protect cells from cisplatin-induced apoptosis, which also implies that cisplatin induction of apoptosis is dependent on an active cdk2.

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