ATR-Chk2 Signaling in p53 Activation and DNA Damage Response during Cisplatin-induced Apoptosis*

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Cisplatin is one of the most effective anti-cancer drugs; however, the use of cisplatin is limited by its toxicity in normal tissues, particularly injury of the kidneys. The mechanisms underlying the therapeutic effects of cisplatin in cancers and side effects in normal tissues are largely unclear. Recent work has suggested a role for p53 in cisplatin-induced renal cell apoptosis and kidney injury; however, the signaling pathway leading to p53 activation and renal apoptosis is unknown. Here we demonstrate an early DNA damage response during cisplatin treatment of renal cells and tissues. Importantly, in the DNA damage response, we demonstrate a critical role for ATR, but not ATM (ataxia telangiectasia mutated) or DNA-PK (DNA-dependent protein kinase), in cisplatin-induced p53 activation and apoptosis. We show that ATR is specifically activated during cisplatin treatment and co-localizes with H2AX, forming nuclear foci at the site of DNA damage. Blockade of ATR with a dominant-negative mutant inhibits cisplatin-induced p53 activation and renal cell apoptosis. Consistently, cisplatin-induced p53 activation and apoptosis are suppressed in ATR-deficient fibroblasts. Downstream of ATR, both Chk1 and Chk2 are phosphorylated during cisplatin treatment in an ATR-dependent manner. Interestingly, following phosphorylation, Chk1 is degraded via the proteosomal pathway, whereas Chk2 is activated. Inhibition of Chk2 by a dominant-negative mutant or gene deficiency attenuates cisplatin-induced p53 activation and apoptosis. In vivo in C57BL/6 mice, ATR and Chk2 are activated in renal tissues following cisplatin treatment. Together, the results suggest an important role for the DNA damage response mediated by ATR-Chk2 in p53 activation and renal cell apoptosis during cisplatin nephrotoxicity.

Cisplatin is a highly effective antineoplastic agent that has been widely used for cancer therapy (1, 2). However, the therapeutic efficacy of cisplatin is limited by its toxicity to normal tissues, notably the kidneys (3, 4). In the kidneys, cisplatin induces cell injury and death in renal tubular cells, leading to acute renal failure (3, 4). Indeed, about a quarter of acute renal failure cases are attributable to cisplatin nephrotoxicity (5). Multiple signaling pathways are activated by cisplatin in renal tubular cells (6–14); nevertheless, the mechanism of renal cell death during cisplatin nephrotoxicity remains largely unclear. As a result, effective interventions for renoprotection during cisplatin chemotherapy are currently lacking.

Recent work has suggested a role for p53 signaling in renal cell apoptosis and cisplatin nephrotoxicity (10, 15–17). p53 is activated early during cisplatin treatment and induces the expression of proapoptotic genes, including PLIMA-α (15, 18). Pharmacologic as well as genetic blockade of p53 ameliorates cisplatin-induced renal cell apoptosis in vitro and nephrotoxicity in vivo (15, 17). Despite these findings, the upstream signaling pathway(s) that leads to p53 activation under this pathological condition remains elusive.

A plausible mechanism of p53 activation during cisplatin nephrotoxicity is DNA damage (1, 2). It is known that cisplatin forms covalent bonds with the purine bases in the DNA, primarily resulting in 1,2- or 1,3-intrastrand cross-linking (2, 19, 20). Cross-linking by cisplatin blocks DNA replication and gene transcription and might further result in double strand breaks (2, 19). The consequent genotoxic stress triggers the activation of a signaling cascade, which may lead to p53 phosphorylation and activation.

The major molecular sensors of DNA damage include ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related), and DNA-PK (DNA-dependent protein kinase) (21, 22). In response to DNA damage or genotoxic stress, these protein kinases are recruited to the site of DNA damage, forming nuclear “foci” (22–24). This is followed by recruitment and activation of other signaling molecules, including Chk1 and Chk2, inducing cell cycle arrest or apoptosis (22, 25). Importantly, these protein kinases can phosphorylate and activate p53 (22, 25). Despite the general understanding of DNA damage response, it is not entirely clear how the initial DNA lesion induced by cisplatin is detected and leads to p53 activation and cell death (1, 2). In the case of cisplatin nephrotoxicity, whether a DNA damage response is triggered and how it is involved in p53 activation and subsequent renal cell apoptosis are completely unknown (4). Investigation of the signaling cascades activated by cisplatin in various cell types would advance our understanding of cisplatin toxicity in normal nonmalignant as well as cancerous tissues.

In the current study, we show that ATR, but not ATM or DNA-PK, is activated during cisplatin treatment of renal cells and tissues. ATR further activates Chk2 to induce p53 activa-
tion and apoptosis. These results suggest an important role for the ATR-Chk2 signaling axis in p53 activation and renal cell apoptosis during cisplatin nephrotoxicity.

MATERIALS AND METHODS

Cells

The immortalized rat kidney proximal tubular cell (RPTC)2 line was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH) and maintained as described previously (15, 26). Human embryonic kidney (HEK) cells were maintained in minimal essential medium with 10% horse serum, glutamine, and antibiotics. Normal and ATR-deficient Seckel fibroblasts were obtained from the Coriell Cell Repository and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. Wild type and Chk2-deficient HCT116 cells were cultured in McCoy’s 5A medium as described previously (27, 28).

Antibodies and Special Reagents

Antibodies were from the following sources: rabbit polyclonal anti-ATM, goat polyclonal anti-ATR, rabbit polyclonal anti-ATRIP, goat polyclonal anti-Rad9, rabbit polyclonal anti-Hus1, rabbit polyclonal anti-Rad1, and rabbit polyclonal anti-DNA-PK antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit polyclonal anti-p53, anti-phospho-p53, anti-Chk1, anti-Chk2, anti-phospho-Chk1, anti-phospho-Chk2, anti-RPA70, and anti-phospho-H2AX antibodies from Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-β-actin antibody from Sigma; mouse monoclonal anti-cytochrome c from BD PharMingen; rabbit polyclonal anti-PUMA from Dr. Yu at the University of Pittsburgh; all secondaries antibodies from Jackson ImmunoResearch (West Grove, PA). Recombinant p53 was purchased from Santa Cruz Biotechnology. Carbobenzoxy-DEVD-7-amino-4-trifluoromethyl coumarin and 7-amino-4-trifluoromethyl coumarin for caspase assay were purchased from Enzyme Systems Products (Dublin, CA).

In Vitro and in Vivo Models of Cisplatin Nephrotoxicity

Cisplatin treatment of cultured cells was conducted as described earlier (15, 18, 29). Cisplatin was used at 20 μM for RPTC cells, 40 μM for HEK cells, 100 μM for normal and ATR-deficient fibroblasts, and 50 μM for HCT116 cells. After cisplatin treatment, cells were morphologically analyzed or harvested to collect cell lysates for various biochemical analyses. For in vivo study, male C57BL/6 mice of 8 weeks purchased from Jackson Laboratory were injected intraperitoneally with a single dose of 30 mg/kg cisplatin to induce kidney injury as previously (30–32). All animal work was performed in accordance with the animal use protocol approved by the Institutional Animal Care and User Committee of the Medical College of Georgia and Veterans Affairs Medical Center at Augusta.

ATR-Chk2 Signaling in Cisplatin-induced Apoptosis

Gene Transfection

Dominant negative ATR, Chk1, and Chk2 containing active site mutations (kinase-dead) were described previously (28, 33–35). RPTC and HEK cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen). RPTC cells had relatively low (~20–30%) transfection efficiency. To identify the transfected cells, green fluorescent protein (GFP) was co-transfected with the target gene at a ratio of 1:5. The subsequent examination was focused on the GFP-labeled (transfected) cells. HEK cells showed a high (over 80%) transfection efficiency; thus, they were used for biochemical and whole cell population analyses to determine the effects of transfected genes. As a control, empty vectors were used for transfection.

In Vitro Immunocomplex Kinase Assay

Renal tissues and cells were lysed with the immunoprecipitation lysis buffer in the presence of protease and phosphatase inhibitors as described previously (36). The lysates were subjected to immunoprecipitation using antibodies specific for ATM, ATR, DNA-PK, or Chk2. The resultant immunoprecipitates were added to a protein kinase reaction containing 20 μM ATP and 12.5 ng/μl recombinant p53 as the phosphorylation substrate. After a 20-min incubation at 30 °C, 2% SDS was added to terminate the reaction. The reaction samples were then subjected to gel electrophoresis and immunoblot analysis to detect the levels of p53 phosphorylation to indicate the protein kinase activity of various protein kinases immunoprecipitated from the cells and tissues.

Co-immunoprecipitation

Cells were lysed with the immunoprecipitation lysis buffer in the presence of protease and phosphatase inhibitors and then subjected to immunoprecipitation as described previously (36, 37). The immunoprecipitates were resuspended in SDS buffer for gel electrophoresis, followed by immunoblot analysis using specific antibodies against various proteins.

Immunoblot Analysis

Protein concentration in various cell lysates was determined by using the bicinchoninic acid reagent from Pierce. Equal amounts (usually 10 μg) of protein were loaded in each lane for electrophoresis. The proteins were then transferred onto polyvinylidene difluoride membranes. The blots were then incubated in a blocking buffer and then exposed to the primary antibodies overnight at 4 °C, followed by the horseradish peroxidase-conjugated secondary antibody. Antigens on the blots were revealed using the enhanced chemiluminescence kit from Pierce.

Dual Immunofluorescence of ATR and Phosphorylated H2AX

RPTC cells were grown on collagen-coated glass coverslips. After cisplatin treatment, the cells were fixed with 4% paraformaldehyde and then permeabilized with 0.4% Triton X-100 in blocking buffer (2% bovine serum albumin, 0.2% milk, and 2% normal goat serum in phosphate-buffered saline). The cells were subsequently exposed with primary antibodies (rabbit anti-phosphorylated H2AX and goat anti-ATR), followed by
incubation with a mixture of fluorescein isothiocyanate-labeled goat-anti-rabbit and Cy3-labeled donkey-anti-goat secondary antibodies. After three washes, signals were examined by confocal microscopy using Cy3 and fluorescein isothiocyanate channels.

**Examination of Apoptosis**

**Morphological Examination**—For morphological examination, untreated or treated cells were stained with 10 μg/ml Hoechst 33342 for 2–5 min. Phase-contrast and fluorescence microscopy were then used to examine the cellular and nuclear morphology. Cells undergoing apoptosis showed cellular shrinkage, nuclear condensation and fragmentation, and formation of apoptotic bodies. Four fields with ~200 cells/field were checked in each group to quantify the percentage of apoptotic cells.

**Caspase Assay**—A caspase assay was used as a biochemical marker of apoptosis as described previously (29). Briefly, cellular extracts by 1% Triton X-100 were added to an enzymatic reaction with 50 μM carboxenzoxy-DEVD-7-amino-4-trifluoromethyl coumarin, a fluorogenic peptide substrate of caspases. After 1 h of reaction at 37 °C, fluorescence was measured at excitation 360 nm/emission 530 nm. A nanomolar amount of liberated 7-amino-4-trifluoromethyl coumarin indicates the caspase activity in the given sample.

**Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Staining**—Annexin V-fluorescein isothiocyanate/propidium iodide staining was performed using a kit from BD Pharmingen as described recently (29). Briefly, HEK cells were detached by trypsinization and harvested by centrifugation at 1,000 g for 5 min. The cells were then resuspended in binding buffer at a density of 1–2 × 10^6 cells/ml. The single cell suspension of 100 l (1–2 × 10^5 cells) was incubated with 5 l of Annexin V-fluorescein isothiocyanate and 5 l of propidium iodide for 15 min at room temperature. Finally, the mixture was diluted with 400 l of binding buffer and analyzed with a FACSCalibur flow cytometer (BD Biosciences). For each sample, total of 10,000 events were counted.

**Analysis of Cytochrome c Release**

Cytosolic and membrane-bound organellar fractions were separated by using digitonin, which at low concentrations selectively permeabilizes the plasma membrane without solubilizing intracellular organelles, including mitochondria (18, 37). Briefly, the cells were incubated with 0.05% digitonin in an isotonic buffer (250 mM sucrose, 10 mM Hepes, 10 mM KCl, 1.5 mM MgCl_2, 1 mM EDTA, and 1 mM EGTA (pH 7.1)) for 2 min at
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ity was decreased in cisplatin-treated cells (Fig. 1A). Similarly, DNA-PK activity was markedly reduced after 2–4 h of cisplatin treatment. To examine the DNA damage-responsive protein kinases, we collected cell lysate after 0–8 h of cisplatin treatment, and its level increases thereafter (not shown). Together, the results suggest that the observed decreases of ATM and DNA-PK activity during cisplatin treatment were due to inactivation of these two protein kinases. In contrast, we detected a progressive increase of ATR kinase activity during cisplatin treatment (Fig. 1C), whereas total ATR expression remained constant. Semiquantification by densitometry of the blots is shown in Fig. 1D. Clearly, ATM and DNA-PK are inactivated, whereas ATR is activated during cisplatin treatment of RPTC cells (Fig. 1D). To further confirm the activation status of ATM, we treated RPTC cells with either cisplatin or etoposide and analyzed ATM phosphorylation at Ser-1981 (38). As shown in Fig. 1F, cisplatin did not induce significant ATM phosphorylation, whereas etoposide did in the same experiment. To gain insights into ATR activation during cisplatin treatment, we analyzed the formation of the RAD9-RAD1-HUS1 (9-1-1) protein complex, which has been implicated in ATR activation during genotoxic stress (23, 24). ATR was immunoprecipitated from untreated and cisplatin-treated RPTC cells. The resultant immunoprecipitates were analyzed for the presence of various proteins. As shown in Fig. 1F, cisplatin treatment induced co-immunoprecipitation of ATR with all three 9-1-1 proteins. In addition, RPA70 and Rad17 were also detected in this protein complex (Fig. 1F). In contrast, ATM did not form complexes with these proteins during cisplatin treatment (not shown). Together, the results suggest a specific activation of ATR during cisplatin treatment of renal tubular cells.

### Statistical Analyses

Data were expressed as means ± S.D. (n ≥ 3). Statistical analysis was conducted using the GraphPad Prism software (GraphPad, San Diego, CA). The statistical differences between two groups studied were determined by t test. p < 0.05 was considered to indicate significant differences.

### RESULTS

**Activation of ATR, but Not ATM or DNA-PK, during Cisplatin Treatment of Renal Tubular Cells**—Our previous work has established an in vitro model of cisplatin nephrotoxicity using cultured RPTC cells (15). In this model, 20 μM cisplatin induces 50–60% apoptosis in 16 h. Notably, p53 is activated early (2–4 h) following cisplatin treatment, and its level increases thereafter. To examine the DNA damage-responsive protein kinases, we collected cell lysate after 0–8 h of cisplatin treatment and then immunoprecipitated ATM, DNA-PK, and ATR. The immunoprecipitates were subjected to an in vitro kinase assay using recombinant p53 as substrate. Protein kinase activity was indicated by p53 phosphorylation in the assay. As shown in Fig. 1A, the kinase activity of ATM (shown as phosphorylated p53 (P-p53)) was markedly reduced after 2–4 h of cisplatin treatment and became undetectable at 8 h. Similarly, DNA-PK activity was decreased in cisplatin-treated cells (Fig. 1B). Consistent with our recent work (36), total protein levels of ATM and DNA-PK did not change significantly during 0–8 h of cisplatin incubation (Fig. 1, A and B). The results suggest that the observed decreases of ATM and DNA-PK activity during cisplatin treatment were due to inactivation of these two protein kinases. In contrast, we detected a progressive increase of ATR kinase activity during cisplatin treatment (Fig. 1C), whereas total ATR expression remained constant. Semiquantification by densitometry of the blots is shown in Fig. 1D. Clearly, ATM and DNA-PK are inactivated, whereas ATR is activated during cisplatin treatment of RPTC cells (Fig. 1D). To further confirm the activation status of ATM, we treated RPTC cells with either cisplatin or etoposide and analyzed ATM phosphorylation at Ser-1981 (38). As shown in Fig. 1F, cisplatin did not induce significant ATM phosphorylation, whereas etoposide did in the same experiment. To gain insights into ATR activation during cisplatin treatment, we analyzed the formation of the RAD9-RAD1-HUS1 (9-1-1) protein complex, which has been implicated in ATR activation during genotoxic stress (23, 24). ATR was immunoprecipitated from untreated and cisplatin-treated RPTC cells. The resultant immunoprecipitates were then analyzed for the presence of various proteins. As shown in Fig. 1F, cisplatin treatment induced co-immunoprecipitation of ATM with all three 9-1-1 proteins. In addition, RPA70 and Rad17 were also detected in this protein complex (Fig. 1F). In contrast, ATM did not form complexes with these proteins during cisplatin treatment (not shown). Together, the results suggest a specific activation of ATR during cisplatin treatment of renal tubular cells.

**Accumulation of ATR to Nuclear Foci during Cisplatin Treatment**—A critical indication of ATR activation during genotoxic stress is the accumulation of ATR to nuclear foci, where signaling proteins accumulate and interact in response to DNA damage (23, 24). To examine the changes of ATR localization, we conducted immunofluorescence in untreated control RPTC cells, ATR showed a fine staining in the nucleus (Fig. 2A, Untreated cells). After cisplatin treatment, ATR staining became coarse and punctate, showing the typical appearance of nuclear foci (Fig. 2A, Cisplatin treated cells). Importantly, at the nuclear foci, ATR co-localized with phosphorylated H2AX (Fig. 2A, p-H2AX), a known DNA damage response protein and phosphorylation target of ATR. The accumulation of ATR and phosphorylated H2AX to nuclear foci was detected at 2 h of cisplatin treatment (Fig. 2A) and increased thereafter (not shown). By immunoblot analysis, we further confirmed that H2AX was phosphorylated during cisplatin treatment in a time-
dependent manner (Fig. 2B). Notably, H2AX phosphorylation was diminished in ATR-deficient cells but not in ATM-deficient cells (Fig. 2C), suggesting that ATR is the major protein kinase for H2AX phosphorylation at the nuclear foci during cisplatin treatment. Together, these results provide further evidence for an early DNA damage response and ATR activation during cisplatin nephrotoxicity.

Role of ATR in p53 Phosphorylation during Cisplatin Treatment—Cisplatin induces an early p53 activation in renal tubular cells, leading to apoptotic gene expression and apoptosis (15, 16, 18). Our results shown above demonstrated the activation of ATR, but not ATM or DNA-PK, during cisplatin treatment of RPTC cells. With these observations, we hypothesized that the DNA damage response mediated by ATR might contribute significantly to cisplatin-induced p53 activation in renal tubular cells. To test this possibility, we initially examined the effects of dominant-negative ATR (dn-ATR) on p53 activation. RPTC cells were transiently transfected with dn-ATR or a control empty vector. GFP was co-transfected to identify the transfected cells for further examination. After cisplatin treatment, P-p53 was analyzed by immunofluorescence to reveal p53 activation. In untreated control cells, the signal of P-p53 was minimal (Fig. 3A, Untreated). Following cisplatin treatment, the cells that were transfected with empty vector showed strong P-p53 staining (Fig. 3A, Cisplatin treated, Vector + GFP, arrows), whereas the cells transfected with dominant-negative ATR did not (Fig. 3A, Cisplatin treated/dn-ATR + GFP, arrowheads). Of note, p53 activation was not suppressed in untransfected cells in the same dish (Fig. 3A, asterisks). The transfection efficiency in RPTC cells was relatively low, not enough for biochemical analysis of the effects of transfected genes. Thus we subsequently examined the effects of dominant-negative ATR in HEK cells, which had a transfection efficiency of 80–90%. HEK cells were transfected with empty vector or dn-ATR and then treated with cisplatin. As shown in Fig. 3B, cisplatin induced p53 activation or phosphorylation in empty vector-transfected HEK cells but not in the cells that were transfected with dn-ATR, supporting a role for ATR in p53 activation during cisplatin treatment. To further substantiate this conclusion, we compared cisplatin-induced p53 activation in wild-type, ATR-deficient, and ATM-deficient fibroblasts. Clearly, cisplatin-induced p53 phosphorylation was ameliorated in ATR-deficient cells (Fig. 3C, right). In sharp contrast, p53 phosphorylation was not inhibited but slightly increased in ATM-deficient cells (Fig. 3C, left). Collectively, these results suggest that ATR has a critical role in p53 activation during cisplatin treatment.

Genetic Blockade of ATR Ameliorates Cisplatin-induced Apoptosis—Renal tubular cell apoptosis during cisplatin nephrotoxicity is initiated by multiple signaling pathways (6–14),...
which may include genotoxic stress and p53 activation (10, 15–17). Since we demonstrated a role for ATR in p53 activation during cisplatin treatment (Fig. 3), we reasoned that inhibition of ATR might abrogate cisplatin-induced tubular cell apoptosis. To test this possibility, we first examined RPTC cells transiently transfected with dominant-negative ATR or a control vector. Apoptosis was indicated by typical cellular and nuclear morphology. As shown in Fig. 4A, cisplatin induced apoptosis in over 60% of cells in the untransfected or vector-transfected groups, but only in 32% of the cells transfected with dn-ATR. Consistently, ATR-deficient fibroblasts were significantly more resistant to cisplatin-induced apoptosis than wild-type cells (Fig. 4B). ATR deficiency also attenuated cisplatin-induced caspase activation in these cells (Fig. 4C). We further determined the effects of dominant-negative ATR on cisplatin-induced apoptosis in HEK cells by flow cytometry following Annexin V staining. As shown in Fig. 4D, untreated control cells had 2% apoptotic cells that were positive for Annexin V staining. Following cisplatin treatment, 47% cells became apoptotic in the group that was transfected with empty vector. In contrast, apoptosis was suppressed to 22% in the group transfected with dominant-negative ATR (Fig. 4D). Thus, ATR may regulate p53 and contribute significantly to cisplatin-induced apoptosis.

Chk2 Activation and Chk1 Degradation during Cisplatin Treatment and Role of ATR—As one of the most upstream protein kinases activated during genotoxic stress, ATR can phospho-

FIGURE 4. Genetic blockade of ATR ameliorates cisplatin-induced apoptosis. A, RPTC cells were co-transfected with either dn-ATR + GFP or empty vector + GFP. The cells were then treated with 20 μM cisplatin or left untreated for 16 h. The cells were fixed and stained with Hoechst 33342. The cellular and nuclear morphology of transfected cells (with GFP fluorescence) was examined to determine the percentage of apoptosis. B, wild-type and ATR-deficient fibroblasts were treated with 100 μM cisplatin for 24 h. The cells were then fixed and stained with Hoechst 33342 to determine the percentage of apoptosis by morphology. C, wild-type and ATR-deficient fibroblasts were treated with 100 μM cisplatin for 24 h and extracted with Triton X-100 for measurement of caspase activity as described under “Materials and Methods.” Data in A–C are expressed as mean ± S.D. (n = 4). D, HEK cells transfected with dn-ATR or control vector were treated with 40 μM cisplatin for 24 h and then stained with Annexin V. The percentage of Annexin V-positive cells was determined by flow cytometry. Shown are representative results.
results demonstrate an early activation of Chk1 and Chk2 during cisplatin treatment of renal cells. Importantly, ATR appears to be a critical upstream regulator of Chk1 and Chk2 under the experimental condition.

Involvement of Chk2 in p53 Activation during Cisplatin Treatment—Although both Chk1 and Chk2 were phosphorylated early during cisplatin treatment, Chk1 was degraded rapidly, whereas Chk2 showed a continuous phosphorylation or activation (Fig. 5). We therefore focused on Chk2 for its involvement in p53 regulation and cisplatin-induced apoptosis. We first determined Chk2 activation during cisplatin treatment by using the in vitro immunocomplex kinase assay. It was shown that Chk2 was activated time-dependently following cisplatin treatment, starting from 2 h (Fig. 6A). Our next experiment tested the effects of dominant-negative Chk2 (dn-Chk2) on cisplatin-induced p53 activation in RPTC cells. To this end, RPTC cells were transfected with dn-Chk2 or control empty vector; GFP was co-transfected to identify the transfected cells for further examination. p53 phosphorylation or activation was analyzed by immunofluorescence (P-p53). As shown in Fig. 6B, p53 activation during cisplatin treatment was attenuated in RPTC cells that were transfected with dn-Chk2 (arrowheads). Consistently, dn-Chk2 blocked cisplatin-induced p53 phosphorylation in HEK cells, as shown by immunoblot analysis (Fig. 6C). In contrast, dominant negative Chk1 did not diminish p53 phosphorylation (Fig. 6C). We further showed that cisplatin-induced p53 phosphorylation was blocked in Chk2-deficient HCT116 cells (Fig. 6D). Collectively, the results from this series of experiments support a role for Chk2 in p53 regulation during cisplatin treatment.

Effects of Chk2 Inhibition on Cisplatin-induced Apoptosis—We went on to determine the role of Chk2 signaling in cisplatin-induced apoptosis. RPTC cells were co-transfected with GFP and dominant-negative Chk2 or empty vector and then subjected to 16 h of cisplatin incubation. Apoptosis in transfected (i.e. GFP-labeled) cells was evaluated by cellular and nuclear morphology. As shown in Fig. 7A, cisplatin induced 60–70% apoptosis in untransfected or vector-transfected cells but only 26% in the cells transfected with dn-Chk2. Interestingly, apoptosis during cisplatin treatment was slightly increased by the transfection of dn-Chk1 as compared with empty vector transfection (Fig. 7A). Further evidence for a proapoptotic role of Chk2 was shown in experiments using Chk2-deficient HCT116 cells. During cisplatin treatment, the Chk2-deficient cells showed significantly less apoptosis and caspase activation than their wild type counterparts (Figs. 7B and C). In addition, we determined the effects of dominant-negative Chk2 on cisplatin-induced apoptosis in HEK cells by using flow cytometry following Annexin V staining. As shown in Fig. 7D, cisplatin induced 45% apoptosis, which was suppressed to 24% by dn-Chk2. Thus, Chk2 plays an important role in cisplatin-induced apoptosis. Considering our earlier observation of Chk2 regulation by ATR, it is suggested that the ATR-Chk2 signaling axis contributes critically to p53 activation and tubular cell apoptosis during cisplatin nephrotoxicity.

ATR and Chk2 in PUMA-α Induction and Cytochrome c Release during Cisplatin Treatment—Our previous work showed that PUMA-α, a proapoptotic Bcl-2 protein, is induced

| A | B |
|---|---|
| Cisplatin | Cisplatin |
| 0 | 0 | 0 |
| 2 | 4 | 8 |
| 8 (hrs) | 8 (hrs) | +LLnV |
| P-Chk1 | Chk1 | |
| Chk1 | β-actin | |
| Chk2 | |
| P-Chk2 | |
| β-actin | |

**FIGURE 5.** Chk2 activation and Chk1 degradation during cisplatin treatment; Role of ATR. A, RPTC cells were treated with cisplatin for 0–8 h. Whole cell lysate was collected for immunoblot analysis of phosphorylated (threonine 68) Chk2, total Chk2, phosphorylated (serine 317) Chk1, total Chk1, and β-actin. B, RPTC cells were treated with cisplatin in the presence or absence of 25 μM LLnV for 0–8 h. Whole cell lysate was collected for immunoblot analysis of phosphorylated (serine 317) Chk1, total Chk1, and β-actin. C, HEK cells were transfected with either empty vector or dn-ATR. The cells were then treated with cisplatin for the indicated times to collect whole cell lysate for immunoblot analysis. The results show that both Chk1 and Chk2 are phosphorylated in an ATR-dependent manner during cisplatin treatment. Chk1, but not Chk2, is degraded following the phosphorylation.
ATR-Chk2 control PUMA-α induction and consequent mitochondrial pathway of apoptosis.

**Activation of ATR and Chk2 during Cisplatin Nephrotoxicity in Vivo**—Whether cisplatin induces genotoxic stress and a DNA damage response *in vivo* in the kidneys is unknown. To gain some initial information, we used a well characterized mouse model of cisplatin nephrotoxicity (30–32). In this model, intra-peritoneal injection of 30 mg/kg cisplatin into C57BL/6 mice leads to acute kidney injury in 3 days, as demonstrated by higher serum creatinine, blood urea nitrogen, tissue pathology, and tubular apoptosis (30–32). Renal tissues collected on different days of cisplatin treatment were examined for various proteins involved in DNA damage response. As shown in Fig. 9A, total ATM decreased after 2–3 days of cisplatin treatment, whereas no obvious ATM phosphorylation at Ser-1981 was detected. Total ATR did not change significantly. There was a marginal increase in Chk1 phosphorylation at day 1 of cisplatin treatment, but both total and phospho-Chk1 decreased at days 2 and 3. The most impressive induction or activation was shown in Chk2, p53, and PUMA-α (Fig. 9A). Following cisplatin treatment, kidney tissues also had higher levels of H2AX phosphorylation (data not shown). To further confirm the *in vivo* activation of ATR-Ch2, we immunoprecipitated ATM, ATR, or Chk2 for an *in vitro* kinase activity assay. The immunoprecipitates were added to an *in vitro* kinase assay containing recombinant p53 as the phosphorylation substrate. Kinase activity was indicated by phosphorylated p53. As shown in Fig. 9B, ATM activity (indicated by P-p53) was decreased after 2–3 days of cisplatin treatment, suggesting inactivation of this protein kinase during cisplatin nephrotoxicity. In sharp contrast, the activity of both ATR and Chk2 increased on days 2 and 3 of cisplatin treatment (Fig. 9, B and C). These results indicate that a DNA damage response mediated by ATR-Chk2 is indeed activated *in vivo* during cisplatin nephrotoxicity.

**DISCUSSION**

This study has shown the first evidence for an early DNA damage response mediated by ATR and Chk2 during cisplatin nephrotoxicity. Importantly, it has demonstrated a role for ATR/Chk2 signaling in p53 activation and subsequent apopto-
sis. Based on these findings, a signaling cascade is proposed as follows (Fig. 10). DNA damage or genotoxic stress induced by cisplatin leads to a rapid activation of ATR and inactivation of ATM and DNA-PK. ATR then phosphorylates Chk1 and Chk2 and probably also p53. Upon phosphorylation, Chk1 is degraded, but Chk2 is activated to further phosphorylate and activate p53. Subsequently, p53 induces the expression of PUMA-α, which accumulates in mitochondria to activate Bax for outer membrane permeabilization, leading to cytochrome c release, followed by caspase activation and apoptosis (Fig. 10).

As some of the results have been confirmed in fibroblasts and HEK and HCT116 cells, it is suggested that cisplatin may activate some common signaling events in various cell types. Of note, renal tubular cell apoptosis during cisplatin nephrotoxicity involves multiple pathways (3, 4). The proposed pathway is a major signaling pathway for DNA damage response under the pathologic condition and, therefore, is not exclusive of other mechanisms.

The DNA damage response is a highly orchestrated and complex signaling event (24). It comprises sensor proteins that recognize damaged DNA; transducer proteins like ATM, ATR, and DNA-PK that relay and amplify the damage signal; and effector proteins, such as Chk1 and Chk2, that control cell cycle progression, DNA repair, and apoptosis (21, 22, 24). The signaling pathway(s) activated is dependent on the type and extent of DNA damage and also the cell type involved. In this study, we show ATM and DNA-PK are not activated but inactivated during cisplatin treatment (Fig. 1). Our previous work showed that ATM plays a cytoprotective role during cisplatin treatment (36). Consistently, Colton et al. (43) showed recently that in fibroblasts, ATM is activated during cisplatin treatment and is responsible for increased nucleotide excision repair activity, lead-
ing to inhibition of apoptosis. Thus, inhibition of ATM shown in the current study may inactivate a cytoprotective mechanism to facilitate DNA damage and apoptosis. Our results also demonstrate the inactivation of DNA-PK during cisplatin treatment of renal tubular cells, an observation that is consistent with previous results by Turchi et al. (44). More interestingly, although ATM and DNA-PK are inactivated, we show that ATR is activated by cisplatin in renal tubular cells and tissues. The specific ATR activation is indicated not only by a progressive increase of kinase activity but by the accumulation of ATR in nuclear foci, co-localizing with phosphorylated H2AX, a marker of DNA damage (Fig. 2). Of note, the increase of ATR activity during cisplatin treatment is not dramatic, which is in line with the scenario that ATR activation is best illustrated by its accumulation in nuclear foci during genotoxic stress (23).

It is unclear how ATR is specifically activated during cisplatin treatment. Nevertheless, we demonstrate the formation of
ATR-Chk2 Signaling in Cisplatin-induced Apoptosis

the 9-1-1 complex in cisplatin-treated cells. Notably, the 9-1-1 complex is pulled down together with RPA70, Rad17, and ATR, suggesting the formation of a huge multiprotein complex. In contrast, ATM does not complex with these proteins following cisplatin treatment. Thus, it is speculated that the specific ATR activation induced by cisplatin may be related to the unique and complex recruitment and interaction of signaling proteins at DNA damage sites. This scenario is supported by the current understanding of differential DNA damage responses initiated by single or double strand breaks (39, 45). Further investigation in this area should provide a thorough and systematic analysis of the signaling protein complexes that are formed at the sites of DNA lesion within cisplatin-treated cells.

Downstream of ATR, we show that both Chk1 and Chk2 are phosphorylated during cisplatin treatment. Chk1 phosphorylation at serine 317 and serine 345 are believed to be essential for maximal kinase activity (21). Interestingly, we show that early phosphorylation of Chk1 is followed by Chk1 degradation, and both changes are blocked by dominant-negative ATR (Fig. 5). Chk1 degradation following cisplatin treatment is intriguing. It was shown recently that ATR-mediated phosphorylation of Chk1 during camptothecin treatment leads not only to Chk1 activation but also to its degradation via the proteasomal pathway (40). Consistently, we demonstrate evidence for a role of Chk1 during camptothecin treatment leads not only to Chk1 activation but also to its degradation via the proteasomal pathway (40). Consistently, we demonstrate evidence for a role of Chk1 degradation during cisplatin treatment (Fig. 5B). Certainly, the Chk1 degradation following initial activation does not exclude a role for Chk1 in cisplatin-induced DNA damage response and consequent apoptosis. Nevertheless, we show that dominant negative Chk1 does not block p53 activation (Fig. 6C). In addition, cisplatin-induced apoptosis is not attenuated by this Chk1 mutant. Together, the results suggest that Chk1 does not have a major role in the DNA damage response during cisplatin treatment.

Chk2 is not structurally homologous to Chk1 but has some overlapping functions with the latter (46). During genotoxic stress, Chk2 is activated by phosphorylation at threonine 68 (46). Upon phosphorylation, the normally monomeric Chk2 undergoes dimerization, leading to increased kinase activity (46). Chk2 is generally believed to be phosphorylated and activated by ATM (46, 47). However, ATR-dependent activation of Chk2 has also been reported (48). Our current results show that, during cisplatin treatment, Chk2 is phosphorylated at threonine 68 and activated in an ATR-dependent manner (Fig. 5). Notably, we also show that Chk2 is activated in vivo during cisplatin nephrotoxicity. Thus, ATR may phosphorylate and activate Chk2 for signaling during cisplatin-induced genotoxic stress. Functionally, we show that ATR/Chk2 signaling is largely responsible for p53 phosphorylation and activation during cisplatin treatment (Figs. 3 and 6). As a result, inhibition of ATR/Chk2 by dominant-negative mutants or genetic knock-out suppresses p53 activation under the experimental condition (Figs. 3 and 6).

Recent studies from this and other laboratories have suggested a role for p53 in tubular cell apoptosis and cisplatin nephrotoxicity (10–15). Our latest work has further demonstrated that p53-deficient mice are protected from cisplatin-induced nephrotoxicity in vivo; the protection is partial but histologically and functionally significant (17). The current study has now gained a mechanistic understanding of the pathway that leads to p53 activation under the pathologic condition. To further support the significance of the ATR/Chk2 pathway, we have demonstrated that the p53-initiated injurious events, including PUMA-α induction, cytochrome c release from mitochondria, and apoptosis, are all suppressed when ATR or Chk2 is inhibited (Figs. 4 and 7).

It remains to be determined whether and to what extents ATR/Chk2 contribute to the development of renal tissue injury in vivo. In this regard, we have provided supportive evidence for ATR/Chk2 activation in vivo during cisplatin nephrotoxicity (Fig. 9). Is the ATR-Chk2 pathway a good target for renoprotection during cisplatin chemotherapy in cancer patients? Based on our results, it seems likely that blocking ATR/Chk2 may ameliorate renal injury by cisplatin. However, it remains unclear whether this intervention may also alleviate the therapeutic effects of cisplatin in tumors or cancer cells. It was shown recently that ATR knockdown in human colon cancer cell lines dramatically sensitizes the cells to cisplatin, suggesting a cytoprotective role for ATR in cancer cells (49). A recent study further suggested that ATR may play a cytoprotective or pro-survival role in cells without functional p53 (50), a condition characterized for over 50% of cancers. If, as suggested by these studies and our current results, ATR signaling is pro-survival in cancer cells and pro-death in kidneys, then targeting of ATR would offer an effective strategy for renoprotection during cancer therapy with cisplatin and its derivatives.

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