Human Ku Autoantigen Binds Cisplatin-damaged DNA but Fails to Stimulate Human DNA-activated Protein Kinase*

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We have identified a series of proteins based on an affinity for cisplatin-damaged DNA. One protein termed DRP-1 has been purified to homogeneity and was isolated as two distinct complexes. The first complex is a heterodimer of 83- and 68-kDa subunits, while the second complex is a heterotrimer of 350-, 83-, and 68-kDa subunits in a 1:1:1 ratio. The 83- and 68-kDa subunits in each complex are identical. The 83-kDa subunit of DRP-1 was identified as the p80 subunit of Ku autoantigen by N-terminal protein sequence analysis and reactivity with a monoclonal antibody directed against human Ku p80 subunit. The 68-kDa subunit of DRP-1 cross-reacted with monoclonal antisera raised against the Ku minor Kup80 subunit. The 68-kDa subunit of DRP-1 cross-reacted with a monoclonal antibody directed against human by N-terminal protein sequence analysis and reactivity with a monoclonal antibody directed against human Ku p80 subunit. The 68-kDa subunit of DRP-1 cross-reacted with monoclonal antisera raised against the Ku autoantigen p70 subunit. The 350-kDa subunit was identified as DNA-PKcs, the catalytic subunit of the human DNA-activated protein kinase, DNA-PK. DRP-1/Ku DNA binding was assessed in mobility shift assays and competed by cisplatin-damaged DNA. Results indicate that DNA binding was essentially unaffected by cisplatin-DNA adducts in the presence or absence of DNA-PKcs. DNA-PK activity was only stimulated with undamaged DNA, despite the ability of Ku to bind to cisplatin-damaged DNA. The lack of DNA-PK stimulation by cisplatin-damaged DNA correlated with the extent of cisplatin-DNA adduct formation. These results demonstrate that Ku can bind cisplatin-damaged DNA but fails to activate DNA-PK. These results are discussed with respect to the repair of cisplatin-DNA adducts and the role of DNA-PK in coordinating DNA repair processes.

cis-Diaminedichloroplatinum(II) (cisplatin) is a widely prescribed chemotherapeutic agent used in the treatment of cancer. The intracellular target of cisplatin is DNA, and cisplatin-DNA adducts are thought to impart its chemotherapeutic efficacy. Resistance to cisplatin is a common clinical problem and is likely a multifactorial process (1). In addition to increased efflux, decreased influx, and drug sequestration, cellular repair of cisplatin-DNA adducts has been suggested as one mechanism of cisplatin resistance (1). A series of cell lines resistant to cisplatin were generated from ovarian carcinoma cells by in vitro exposure to increasing concentrations of cisplatin (2). The resistant cells were found to have an increased ability to repair both intrastrand and interstrand cisplatin-DNA adducts, supporting the hypothesis that DNA repair contributes to cisplatin resistance (3).

The cellular activities responsible for metabolizing cisplatin-DNA adducts remain largely undefined. In vitro excision repair assays have established that the human excision repair pathway is capable of repairing 1,2-d(GpG)cisplatin intrastrand DNA adducts, albeit at a considerably lower efficiency compared with the repair of thymine dimers (4–6). In addition to intrastrand adducts that are repaired by the nucleotide excision repair pathway, interstrand DNA cross-links are formed in the reaction with cisplatin and are likely to be repaired by a double-stranded DNA break mechanism. In elucidating the specific pathways that mediate repair of cisplatin-DNA adducts, the identification of cellular proteins that have an affinity for cisplatin-damaged DNA has been an active area of research (reviewed in Ref. 7). High mobility group 1 protein (HMG-1) has been identified as a cisplatin-DNA binding protein (8–10), in addition to a series of other proteins that contain HMG boxes (11, 12). The specific role HMG-1 plays in cellular DNA metabolism is still unclear despite first being identified as a non-histone chromosomal protein more than 20 years ago. The human single-stranded DNA binding protein, replication protein A (RPA), has also been identified in cisplatin-DNA-protein complexes by Western blot analysis (13). RPA has also been demonstrated to be required for excision repair of thymine dimers (14) and presumably cisplatin-damaged DNA and may play a role in mediating repair processes.

In this report, we have identified the human Ku autoantigen as a cisplatin-DNA binding protein. Ku has been previously purified and characterized as a DNA binding protein with an affinity for the ends of double-stranded DNA (15–17). More recently Ku was shown to bind to DNA substrates containing small gaps and nicks (18). Ku can also form a complex with the 350-kDa catalytic subunit (DNA-PKcs) of the human DNA-activated protein kinase (DNA-PK) (19). DNA-PKcs is a nuclear serine/threonine protein kinase that requires DNA for activity (reviewed in Refs. 20 and 21). Ku is the DNA binding component, and the Ku-DNA complex stimulates DNA-PKcs protein kinase activity (19). Independently, Ku and DNA-PK protein have been implicated in double-strand break repair and V(DJ) recombination. The radiosensitive cell line xrs-6 deficient for both V(DJ) recombinase and double-strand break repair is devoid of Ku DNA end binding activity (22, 23) and DNA-PK activity (22). Introduction of the gene encoding p86 into xrs-6 cells restored both end binding activity and DNA-PK activity, and the addition of purified Ku to extracts from xrs-6 cells...
restored in vitro DNA-PK activity (22, 23). Another radiation-sensitive cell line belonging to the same complementation group was shown to be deficient in DNA end binding activity and was initially attributed to the loss of the Ku p70 subunit (24). Interestingly, these cells were not rescued by expression of the p70 gene, but were partially rescued by expression of the p86 Ku cDNA (25). Expression of the the gene encoding p86 also restored end binding activity and V(D) recombination (25).

DNA-PK can phosphorylate a number of proteins including Sp1, p53, SV-40 large T antigen, the C-terminal domain of RNA pol II, and the 32-kDa subunit of RPA (for a review see Ref. 20). Recent cloning of the DNA-PK reveals homology to the phosphatidylinositol 3-kinase family, specifically those members involved in cell cycle regulation (26). We have assessed the affinity of Ku for cisplatin-damaged DNA and the ability of Ku involved in cell cycle regulation (26). We have assessed the phatidylinositol 3-kinase family, specifically those members polII, and the 32-kDa subunit of RPA (for a review see Ref. 20).

Interestingly, these cells were not rescued by expression of the p70 gene, but were partially rescued by expression of the p86 Ku cDNA (25). Expression of the the gene encoding p86 also restored end binding activity and V(D) recombination (25).

**DNA-PK Inhibition by Cisplatin-damaged DNA**

**EXPERIMENTAL PROCEDURES**

**Materials—**Unlabeled nucleotides were from Pharmacia Biotech, and radiolabeled nucleotides were from Du Pont NEN. M13mp18 phage was prepared according to Sambrook et al. (27), and RF-DNA was purified using Qiagen columns (Qiagen, Chatsworth, CA) according to the manufacturer’s protocol. Oligonucleotides were synthesized on a Molecular Biosystems 390 DNA synthesizer and purified by 15% polyacrylamide, 7 M urea preparative DNA sequencing gel electrophoresis (27). Poly(dA-dT) was from Midland Certified Reagents (Midland, TX), and Sequenase Version 2.0 was from U. S. Biochemical Corp. Restriction enzymes, immuno reagents, and cell culture supplies were from Life Technologies, Inc. All other reagents were purchased from standard suppliers.

**DNA Substrates—**The DNA oligonucleotides used in this study have been described previously (10). A 75-mer DNA with the sequence 5'-TACCCGGGGATTCCCTTTAGTGAGGGTTAATTCCGAGCT-3' was employed to construct a 32P-labeled fully duplex DNA substrate. The 75-base pair fully duplex DNA substrate was prepared by annealing 10 pmol of the 75-mer with 20 pmol of a 24-mer DNA oligonucleotide (5'-AGCTCGGAATTAACCTCCTACTAAA-3'), heating to 95 °C for 5 min, and cooling to room temperature over the next hour in buffer containing 50 mM Tris-Cl, pH 7.5, 5 mM DTT, and 10 mM magnesium acetate. The DNA was extended and labeled with 100 μM dATP, dCTP, dTTP, and [α-32P]dGTP (3000 Ci/mmol, 100 μCi) using Sequenase. Extension reactions were performed for 30 min and then chased with 100 μM unlabeled dGTP to ensure the 32-mer was fully extended. For the purposes of comparison, for DNA-PK assays, [α-32P]dGTP was replaced with 100 μM unlabeled dGTP.

**DNA Platination—**The 32P-labeled 75-mer DNA substrate was incubated with varying concentrations of cisplatin (Sigma) as described previously (10). The DNA was purified from uncleaved cisplatin by G-50 spin column chromatography (28). The qmp recovered was determined by liquid scintillation counting an aliquot of the duet, and based on the specific activity obtained in the original labeling reactions, the fmol of DNA recovered was calculated.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA were performed as described previously (10). Briefly, reactions contained 50 fmol of [32P]DNA and were allowed to reach equilibrium by incubation on ice for 30 min. Reaction products were separated by native polyacrylamide gel electrophoresis. Gels were dried and the products were visualized by autoradiography and quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). One unit of DNA binding activity equals 50 fmol bound in 20 μl.

DNA-PK Assays—DNA-PK assays were performed in a final volume of 20 μl containing 40 μM Hepes, pH 7.5, 50 mM KCl, 8 μM MgCl2, 0.2 μM EGTA, 1 mM DTT, 2.5% glycerol, [γ-32P]ATP (0.2-0.5 μCi, 325 μM), and 12 μg of dephosphorylated casein (Sigma). Proteins and DNA were added as indicated in the figure legends and reactions incubated at 30 °C. Reactions were terminated by the addition of an equal volume of 30% acetic acid and spotted on Whatman P-81 phospholipase paper. Filters were washed 5 times for 5 min each in 15% acetic acid, once briefly in 100% methanol, dried, and radioactivity quantified in each spot by PhosphorImager analysis.

Cisplatin-DNA-Sepharose—Double-stranded sperm DNA (USB, Cleveland, OH) was digested with EcoRI and BamHI for 18 h, and precipitated with ethanol, and dissolved in 10 mM Tris-Cl, pH 8.0, 1 mM EDTA at a concentration of 5 mg/ml. The digested DNA was treated with cisplatin (Sigma) at a DNA ratio of 0.05:1, for 18 h at 37 °C in the dark. This ratio of cisplatin/DNA results in approximately 1 adduct every 20 bases (29). The DNA was made 1 M NaCl and precipitated with ethanol, washed extensively. DNA was dissolved in 12,000 × g. The DNA pellet was resuspended in TE (Tris-EDTA) and precipitated with sodium acetate and ethanol. The resulting pellet was washed with 70% ethanol and dissolved in carbonate buffer, pH 9, at a concentration of 2 mg/ml. DNA was coupled to CNBr-activated Sepharose-4B (Pharmacia) according to the manufacturer’s protocol. The final gel preparation contained 500 μg of DNA/mg of resin.

**Protein Purification—**Cell-free extracts were prepared from 4 liters of HeLa cells (2.5 × 10^9 cells) as described previously (10) and fractionated on a 60-ml cisplatin-DNA Sepharose column equilibrated in buffer A (50 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 20% glycerol) containing 0.1 mM NaCl and washed extensively. All buffers used in protein purification were supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin. DRP-I cisplatin-DNA binding activity was eluted in buffer A containing 0.5 mM NaCl. The eluate was diluted to a conductivity equal to 100 mM NaCl and applied to a 15-ml Whatman P-11 phosphocellulose column. Protein was eluted with a linear gradient of buffer B (50 mM NaCl, Gradient). Protein was again eluted with a 40-ml linear gradient from 0.1 to 1 M NaCl buffer A. Two separate peaks of binding activity termed DRP-1A and DRP-1B were obtained and pooled separately. Each pool was diluted to a conductivity equivalent to 100 mM NaCl and fractionated on a 5-ml Q-Sepharose column. Bound protein was eluted with a 40-ml linear gradient from 0.1 to 1 M NaCl in buffer A, and fractions containing at least 50% of the maximum activity were pooled. The DNA binding activity of DRP-1A eluted from the Q-Sepharose column with 250 mM NaCl and DRP-1B eluted with 225 mM NaCl. DRP-1A was highly purified following Q-Sepharose chromatography and further fractionation resulted in a reduction in specific activity (data not shown). The Q-Sepharose pool of DRP-1B still contained multiple proteins and, therefore, was fractionated further. The DRP-1B was diluted as described earlier and applied to a 5-ml heparin-Sepharose column equilibrated in buffer A containing 0.1 M NaCl. Bound protein was then eluted with a 40-ml linear gradient from 0.1 to 1 M NaCl in buffer A. Two-milliliter fractions were collected, assayed for cisplatin-DNA binding activity, and peak fractions pooled. The final step in the purification of DRP-1B was chromatography on a 1-ml S-Sepharose column. The heparin-Sepharose pool was diluted in buffer B (50 mM KCl, 20% glycerol, 1 mM EDTA) to a conductivity equal to 0.1 M KCl and applied to a 1-ml S-Sepharose column equilibrated in buffer B containing 0.1 M KCl. A 10-ml linear gradient from 0.1 to 1 M KCl was employed to elute activity. 0.5-ml fractions were collected, assayed for binding activity, pooled as described above, and frozen at −70 °C.

**Protein Analysis—**SDS-PAGE was performed according to Lammeli (30), and gels were stained with silver according to Morrissey (31). Proteins were transferred to Immobilon P (Millipore, Bedford, MA) using electroblotting transfer in 50 mM CAPS, pH 11, 10% methanol at 350 mA for 3.5 h at 4 °C. Membranes were blocked for 2 h in blocking buffer (2% non-fat dry milk, 0.1% Tween 20 in phosphate-buffered saline). Monoclonal Ab (mAb) 111, directed against the Ku p80 subunit, and mAb N3H10, directed against the Ku p70 subunit, were provided by Dr. Westley Reeves (University of North Carolina) and were diluted 1:100 in blocking buffer (17, 32). Membranes were incubated with primary antibody for 1 h at room temperature and washed 3 times for 15 min with phosphate-buffered saline. Membranes were then incubated with a 1:1000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate secondary-antibody for 1 h. Membranes were washed as described above, visualized using chemiluminescent detection (Du Pont NEN). For automated N-terminal sequence analysis, proteins were separated by SDS-PAGE and transferred to Immobilon P as described above. Following transfer, the membrane was stained with 0.1% Coomassie Blue in 50% methanol, destained in 50% methanol, and the protein bands of interest excised. The membrane pieces were rinsed briefly in 100% methanol, dried, and subjected to automated Edman degradation. Results

We have purified a protein capable of binding duplex DNA substrates damaged with cisplatin. We previously identified two activities capable of binding to cisplatin-damaged DNA duplex substrates following fractionation of HeLa whole cell extracts on a cisplatin-DNA-Sepharose column matrix. The
protein responsible for one of the protein-DNA complexes was identified as HMG-1 (10). We then set out to purify the proteins involved in formation of the other complex, termed DRP-1. The purification of DRP-1 is detailed under "Experimental Procedures." Following initial fractionation on a cisplatin-DNA-Sepharose column, the eluate was fractionated on a phosphocellulose column as described under "Experimental Procedures." DRP-1 activity was eluted with a linear gradient from 0.1 to 1 M NaCl, and each fraction was assayed for cisplatin-DNA binding in an EMSA. An autoradiogram of the EMSA is shown in Fig. 1 and demonstrates two peaks of DRP-1 DNA binding activity. The DRP-1 DNA-protein complexes observed upon native gel electrophoresis in fractions 7 and 9 are nearly identical. Fraction 7, termed DRP-1A, eluted at a NaCl concentration of 200 mM whereas fraction 9, termed DRP-1B, eluted at 300 mM NaCl. The slower migrating DRP-1 DNA-protein complex is likely a supershift representing two protein molecules bound to a single DNA substrate. In addition, a third complex running as a diffuse band near the top of the gel is observed in fractions 8 and 9. The faster migrating complex eluting mainly in fractions 11 and 12 is the HMG-1-cisplatin-DNA complex (10). DRP-1A and DRP-1B were collected and processed separately throughout the remaining steps in the purification as described under "Experimental Procedures." Results of the purification of DRP-1 are summarized in Table I.

The pooled fractions from each column were analyzed by SDS-PAGE (Fig. 2). Protein subunits of 83- and 68-kDa were described under "Experimental Procedures." Resultsofthepurificationof200 mM whereas fraction 9, termed DRP-1B, eluted at a NaCl concentration of 200 mM whereas fraction 9, termed DRP-1B, eluted at 300 mM NaCl. The slower migrating DRP-1 DNA-protein complex is likely a supershift representing two protein molecules bound to a single DNA substrate. In addition, a third complex running as a diffuse band near the top of the gel is observed in fractions 8 and 9. The faster migrating complex eluting mainly in fractions 11 and 12 is the HMG-1-cisplatin-DNA complex (10). DRP-1A and DRP-1B were collected and processed separately throughout the remaining steps in the purification as described under "Experimental Procedures." Results of the purification of DRP-1 are summarized in Table I.

The pooled fractions from each column were analyzed by SDS-PAGE (Fig. 2). Protein subunits of 83- and 68-kDa were selectively concentrated in the DRP-1A column pools (lanes 1-4). The final fraction of DRP-1A contained only the 83- and 68-kDa protein suggesting a heterodimeric structure. SDS-PAGE analysis of the DRP-1A column fractions obtained from Q-Sepharose chromatography demonstrated that the 83- and 68-kDa proteins co-migrated with cisplatin-DNA binding activity (data not shown). Interestingly, highly purified DRP-1B contained three predominant proteins. In addition to the 83- and 68-kDa subunits observed in DRP-1A, a protein migrating greater than the 220-kDa standard is also observed in DRP-1B fractions (Fig. 2, lanes 5-8). These three proteins co-migrated with cisplatin-DNA binding activity in DRP-1B fractionation on the Q, heparin, and S-Sepharose columns (data not shown). Quantification of the intensity of each band suggests that the high molecular mass, 83- and 68-kDa, proteins are present in a 1:1:1 stoichiometry (data not shown). The 83- and 68-kDa subunits present in both DRP-1A and -B likely account for their similar migration pattern observed upon EMSA analysis (Fig. 1). The diffuse high molecular weight complex observed in phosphocellulose fractions 8 and 9 and in the Q-Sepharose B pool (data not shown) may be a trimeric complex consisting of the high molecular weight protein, in addition to the p68 and p83 subunits. In addition, DRP-1B has a specific activity approximately 30% of that obtained for DRP-1A (Table I). Results from the SDS gel indicate that this lower specific activity is likely the result of the large subunit of DRP-1B contributing to the protein level without affecting DNA binding. The net result being a decreased specific activity compared with DRP-1A pools.

N-terminal sequence analysis of the purified proteins was performed to identify the components of DRP-1. DRP-1 fractions were separated by 10% SDS-PAGE and processed for automated N-terminal sequence analysis as described under "Experimental Procedures." The 68-kDa subunit was blocked at the N terminus and yielded no sequence information. Reliable N-terminal sequence data were obtained from the p83
subunit. The first four cycles yielded ambiguous data; however, the following 12 cycles revealed unambiguous sequence information (Table II). A BLAST sequence homology search revealed identity in 11 out of 12 amino acids to the p80 subunit of human Ku autoantigen. Ku is a heterodimer of 80- and 70-kDa subunits that has DNA binding activity (15, 17). It also has been reported that the 70-kDa subunit is acetylated at the N terminus which would account for the inability to obtain N-terminal sequence data. These results strongly suggest that the activity binding to cisplatin-damaged DNA is Ku autoantigen. To confirm that in fact DRP-1 consists of Ku subunits, monoclonal antibodies generated against purified human Ku p80 and p70 (17, 32) were used to probe Western blots of the fractions obtained during the purification. The DRP-1B pools from each step during the purification were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The filter was first probed with mAb N3H10 (anti-Ku p70) and visualized following secondary antibody as described under “Experimental Procedures.” The data obtained demonstrated that the p68 protein cross-reacted with mAb N3H10 (1.1 μg); lane 5, heparin-Sepharose B (0.85 μg); lane 6, S-Sepharose B (0.85 μg). The position of molecular weight markers and mass in kDa is indicated.
DNA-PK Inhibition by Cisplatin-damaged DNA

We have further characterized the interaction of cisplatin with DRP-1/Ku and the activation of DNA-PK. The 75-mer was treated with intermediate cisplatin concentrations, and the resulting damaged DNA was assessed with respect to the ability to stimulate DNA-PK and Ku binding. EMSA analyses showed no significant difference in binding DNA treated at D/N ratios of 0.01:1 and 0.1:1 (data not shown). Interestingly, we observed varying levels of DNA-PK stimulation which inversely correlated with the degree of DNA damage (Fig. 7A). The lowest concentration of cisplatin employed (D/N 0.01:1) results in at least 1 adduct on each DNA as assessed by exonuclease digestion (10) (data not shown). These results suggest that DNA substrates containing multiple cisplatin-DNA adducts inhibit DNA-PK stimulation to a greater extent than DNA substrates with fewer adducts. One explanation is that high levels of cisplatin-DNA adducts may serve to sequester Ku in an inactive state. To address this point, an experiment was performed to determine the ability of Ku to dissociate from a cisplatin-damaged DNA substrate, bind an undamaged DNA, or undamaged DNA in the absence of ATP. The DNA-PK reactions were then initiated with the addition of either ATP or ATP plus poly(dA·dT). The results demonstrate that the DNA-PK activity of DRP-1 B preincubated with either a buffer control without DNA, cisplatin-damaged DNA, or undamaged DNA in the absence of ATP. The DNA-PK reactions were then initiated with the addition of either ATP or ATP plus poly(dA·dT). The results demonstrate that the DNA-PK activity of DRP-1 B preincubated with either a buffer control without DNA, cisplatin-damaged DNA, or undamaged DNA in the absence of ATP. The DNA-PK reactions were then initiated with the addition of either ATP or ATP plus poly(dA·dT). The results demonstrate that the DNA-PK activity of DRP-1 B preincubated with either a buffer control without DNA, cisplatin-damaged DNA, or undamaged DNA in the absence of ATP.

Fig. 6. DNA-PK activity of DRP-1 with cisplatin-damaged and undamaged DNA. A, mock-treated (filled circles) or cisplatin-treated at a 1:1 D/N ratio (open circles) fully duplex 75-mer was assessed for stimulation of DNA-PK activity using 32 ng of DRP-1 B fraction V. Varying concentrations of DNA were preincubated with DRP-1 in the absence of ATP for 30 min on ice. Reactions were initiated with [γ-32P]ATP and incubated at 30°C for 15 min. Reactions were terminated and processed as described under "Experimental Procedures." B, increasing concentrations of DRP-1 were incubated with 7 nM DNA, either mock-treated (filled circles) or treated with cisplatin at a 1:1 D/N ratio (open circles). Reactions were performed as described above. Each point represents the average and standard deviation of duplicate determinations.

Fig. 5. EMSA analysis of DRP-1 binding to cisplatin-damaged DNA. The double-stranded 75-mer DNA was constructed as described under "Experimental Procedures" and mock-treated (lanes 1-9) or treated with cisplatin at a D/N ratio of 1:1 (lanes 10-10). EMSAs were performed using the indicated amount of fraction VIB and 50 fmol of DNA in the absence of competitor DNA. Lanes 1 and 6, without added DRP-1; lanes 2 and 7, 2.75 ng; lanes 3 and 8, 5.5 ng; lanes 4 and 9, 11 ng; lanes 5 and 10, 22 ng. The position of free and bound DNA is indicated. (data not shown). The EMSA results were confirmed in a series of competition binding assays. The first series employed the 32P-labeled 75-mer and increasing concentrations of undamaged M13 RF-DNA that was digested with HhaI. HhaI digestion results in 26 fragments ranging in size from 8 to 877 base pairs, a high concentration of DNA ends which compete for Ku binding and decreases the observed shift of the 32P-labeled 75-mer DNA. The DNA bound was quantified, and results demonstrate that the cisplatin-damaged DNA was bound slightly less avidly than the undamaged DNA (data not shown). The second series of competition experiments employed cisplatin-damaged M13 RF-DNA as a competitor. A vast excess of M13 RF-DNA was required to compete binding to the linear duplex labeled DNA, consistent with the end binding activity of Ku. The degree of competition observed with the cisplatin-damaged DNA was only marginally less than that observed with the undamaged DNA (data not shown). In addition, the DNA substrate employed in the EMSA contains potential cisplatin adduct sites within 10 base pairs of each termini. Considering the end binding activity of Ku it is unlikely that the position of Ku on the damaged DNA spans a region not containing a cisplatin adduct. These results all support the conclusion that Ku retains its ability to bind DNA ends independent of cisplatin-damage.

Since Ku is able to bind cisplatin-damaged DNA we expected activation of DNA-PK to be similar to that obtained using undamaged DNA as a cofactor. The 75-mer DNA substrates were prepared without the 32P label, treated with cisplatin at a D/N ratio of 1:1, and used to assess DNA-PK activity in DRP-1 fraction V. The results of DNA titration experiments (Fig. 6A) demonstrate that the cisplatin-damaged DNA does not activate DNA-PK. The level of kinase activity at maximum stimulation using cisplatin-DNA is approximately 10% of the level observed with undamaged DNA. Titration of DRP-1 fraction V in reaction with constant DNA (Fig. 6B) revealed a similar trend with cisplatin-damaged DNA activating at a level approximately 10% of that observed for the undamaged control DNA. These data and those in Fig. 5 collectively demonstrate that Ku antigen can bind cisplatin-damaged DNA but is unable to activate DNA-PK. While the affinity of Ku for undamaged and cisplatin-damaged DNA is similar, there is a fundamental difference in the ability of Ku bound to cisplatin-DNA to activate DNA-PK.

We have further characterized the interaction of cisplatin with DRP-1/Ku and the activation of DNA-PK. The 75-mer was treated with intermediate cisplatin concentrations, and the resulting damaged DNA was assessed with respect to the ability to stimulate DNA-PK and Ku binding. EMSA analyses showed no significant difference in binding DNA treated at D/N ratios of 0.01:1 and 0.1:1 (data not shown). Interestingly, we observed varying levels of DNA-PK stimulation which inversely correlated with the degree of DNA damage (Fig. 7A). The lowest concentration of cisplatin employed (D/N 0.01:1) results in at least 1 adduct on each DNA as assessed by exonuclease digestion (10) (data not shown). These results suggest that DNA substrates containing multiple cisplatin-DNA adducts inhibit DNA-PK stimulation to a greater extent than DNA substrates with fewer adducts. One explanation is that high levels of cisplatin-DNA adducts may serve to sequester Ku in an inactive state. To address this point, an experiment was performed to determine the ability of Ku to dissociate from a cisplatin-damaged DNA substrate, bind an undamaged DNA, and then stimulate DNA-PK activity. The results shown in Fig. 7B suggest that this is in fact the case. DRP-1B was preincubated with either a buffer control without DNA, cisplatin-damaged DNA, or undamaged DNA in the absence of ATP. The DNA-PK reactions were then initiated with the addition of either ATP or ATP plus poly(dA·dT). The results demonstrate that the DNA-PK activity of DRP-1 B preincubated with either a buffer control without DNA, cisplatin-damaged DNA, or undamaged DNA in the absence of ATP. The DNA-PK reactions were then initiated with the addition of either ATP or ATP plus poly(dA·dT). The results demonstrate that the DNA-PK activity of DRP-1 B preincubated with cisplatin-damaged DNA and initiated with ATP and poly(dA·dT) stimulate DNA-PK to the same extent as DRP-1/Ku preincubated in the absence of DNA. These results suggest that DRP-1 can freely dissociate from cisplatin-damaged DNA to bind undamaged DNA, therefore stimulating DNA-PK activity. Preincubation of DRP-1 with undamaged DNA resulted in a greater level of...
DNA-PK activity independent of poly(dA-dT) in the initiation mix. This is the result of the increased DNA concentrations and the effect of pre-binding DRP-1 to a DNA substrate which is active in stimulating DNA-PK activity. These results demonstrated that DRP-1/Ku can bind cisplatin-damaged DNA but does not sequester Ku on the damaged DNA. An alternative mechanism is that highly damaged DNA substrates allow Ku binding but block the association of the large DNA-PKcs sub-unit as a result of cisplatin-induced DNA distortions. This mechanism is unlikely, based on a series of experiments. The results presented in Fig. 1 show evidence for a trimeric complex in fractions containing Ku and DNA-PKcs. The formation of this trimeric complex on cisplatin-damaged DNA argues against the mechanism of inhibition being the inability of DNA-PKcs to associate with Ku. Additional experiments confirm the conclusion that DNA-PKcs can form a complex with Ku bound to cisplatin-damaged DNA (discussed below).

**DISCUSSION**

In this report we have purified Ku and DNA-PK as proteins having an affinity for cisplatin-damaged DNA. All results presented in this report utilized casein as a substrate for DNA-PK; however, DRP-1B can phosphorylate a synthetic peptide based on the transactivation domain of p53, which has been shown to be specific to DNA-PK (41). We have also demonstrated two intracellular pools of Ku, one free and one apparently complexed with DNA-PKcs, to form DNA-PK. Previous reports have demonstrated that DNA-PKcs and Ku assemble on double-stranded DNA, and the removal of DNA led to the dissolution of the complex (34). At this point we cannot rule out the possibility that low levels of small DNA fragments are present and stabilize the complex of Ku with DNA-PKcs. The finding that Ku was able to bind DNA highly damaged with cisplatin prompted us to assess the ability of cisplatin-damaged DNA to stimulate DNA-PK activity. We demonstrated that in fact DNA damaged with cisplatin is unable to stimulate DNA-PK activity and results in a diminution of kinase activity dependent on the degree of DNA damage. This was a surprising result as most all other DNA structures that have been demonstrated to bind Ku also serve to activate DNA-PK (16, 18). The only other DNA that fails to activate DNA-PK while being bound to Ku is single-stranded DNA (19, 35). The inability of cisplatin-damaged DNA to stimulate DNA-PK activity could be the result of the formation of a single-stranded DNA but is unlikely as cisplatin adducts can cause distortions in the DNA structure including bending, unwinding, and localized denaturation, all with minimal generation of single-stranded DNA (36–40). In addition, the interstrand cross-links observed in DNA treated with cisplatin at D/N ratios of 0.1:1 and 1:1 promote the retention of double-stranded structure, and these DNA substrates show less activation of DNA-PK compared with DNA substrates with minimal interstrand cross-links (Fig. 7A). The ability of DNA-bound Ku to stimulate DNA-PK occurs via an interaction with DNA-PKcs. One mechanism of cisplatin-DNA inhibition of DNA-PK is the Ku-cisplatin-DNA complex does not physically associate with DNA-PKcs and therefore does not stimulate kinase activity. This mechanism is unlikely based on the formation of a trimeric complex in fractions containing Ku and DNA-PKcs observed in Fig. 1. In addition, cross-linking studies show that the DNA-PKcs can be UV cross-linked to cisplatin-damaged DNA. This result provides direct evidence that DNA-PKcs can form a complex with cisplatin-damaged DNA. DNA-PK has been reported to catalyze autophosphorylation of all three subunits, and autophosphorylation of the 350-kDa subunit decreases kinase activity (20). Cisplatin-DNA-dependent autophosphorylation of DNA-PKcs also could account for the decreased kinase activity observed with cisplatin-damaged DNA using exogenous kinase substrates. In preliminary experiments we have demonstrated autophosphorylation of DNA-PKcs in reactions containing cisplatin-damaged DNA. The mechanism by which cisplatin-damaged DNA inhibits DNA-dependent stimulation of DNA-PK activity is actively being pursued.

The observation that cisplatin-damaged DNA fails to activate DNA-PK has implications in the regulation of repair of cisplatin-DNA adducts. DNA-PK is thought to coordinate nuclear events by the phosphorylation of a variety of transcription factors and has been implicated in DNA repair, replication, and recombination (20). The DNA structures recognized by Ku, DNA ends and single-strand to double-strand transitions are likely to be generated during the repair of damaged DNA. Binding of Ku and DNA-PK activation then results in the phosphorylation of a number of proteins and, specifically, transcription factors. The implications are that phosphorylation of these factors may regulate transcriptional processes. p53 has been shown to be phosphorylated on serine residues in the N-terminal transactivation domain by DNA-PK (41), which has been implicated in the stability of p53 (42). One hypothesis is that the increased stability of p53 would result in the induction of a cell cycle check point, which would allow time to repair damaged DNA. The fact that cisplatin-damaged DNA fails to activate DNA-PK would result in p53 stability being unaffected and cell cycle progression unaltered including entry into S-phase. The presence of cisplatin-DNA adducts inhibits purified DNA-PK activity.
DNA replication proteins (43, 44), in addition to in vitro DNA replication and S-phase progression in vivo (45, 46). Cisplatin-induced inhibition of S-phase progression results in the induction of apoptosis (47, 48). DNA-PK has also been reported to phosphorylate RPA (49, 50). In vitro, the extent of RPA phosphorylation had no effect on DNA replication or DNA repair reactions (51). However, UV light-induced cell cycle arrest has been correlated with the degree of RPA phosphorylation and, specifically, a hyperphosphorylated form of RPA (52). One explanation is that UV-induced DNA damage activates DNA-PK and hyperphosphorylation of RPA which contributes to cell cycle arrest. One might expect that UV-damaged DNA would still activate DNA-PK activity. This cascade of events, while clearly speculative and currently without experimental support, could account for the toxicity of cisplatin, its clinical effectiveness, and low rate of repair. This avenue of research is currently under investigation.

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REFERENCES

1. Perez, R. P., Hamilton, T. C., Ozols, R. F., and Young, R. C. (1993) Cancer 71, 1571–1580.
2. Masuda, H., Ozols, R. F., Lai, G. M., Fojo, A., Rothenberg, M., and Hamilton, T. C. (1998) Cancer Res. 48, 5713–5716.
3. Johnson, S. W., Perez, R. P., Godwin, A. K., Yeung, A. T., Handel, L. M., Ozols, R. F., and Hamilton, T. C. (1994) Biochem. Pharmacol. 47, 689–697.
4. Sibghat-Ullah, I., Husain, I., and Sancar, A. (1989) Nucleic Acids Res. 17, 4471–4484.
5. Jones, S. L., Hickson, I. D., Harris, A. L., and Harnett, P. R. (1994) Int. J. Cancer 59, 388–393.
6. Jones, S. L., and Harnett, P. R. (1994) Biochem. Pharmacol. 48, 1662–1665.
7. Chu, G. (1994) J. Biol. Chem. 269, 787–790.
8. Hughes, E. N., Engelsberg, B. N., and Billings, P. C. (1992) J. Biol. Chem. 267, 13520–13527.
9. Pil, P. M., and Lippard, S. (1983) Science 256, 234–237.
10. Turchi, J. J., Li, M., and Henkels, K. M. (1996) Biochemistry 35, 2992–3000.
11. Brown, S. J., Kellett, J. P., and Lippard, S. J. (1997) Science 261, 603–605.
12. Boubnov, N. V., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamini, D. M., Pulaski, C. R., Band, H., Reeves, W., Hendrickson, E. A., and Weaver, D. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 890–894.
13. Smider, V., Rathmell, W. K., Liebe, M. R., and Chu, G. (1994) Science 266, 288–291.
14. Hartley, K. O., Gell, D., Smith, C. G., Zhang, H., Divena, N., Connelly, M. A., Adron, A., Lees-Miller, S. P., Anderson, C. W., and Jackson, S. P. (1995) Cell 82, 849–856.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY.
16. Penefsky, H. S. (1997) J. Biol. Chem. 252, 2891–2899.
17. Ushay, H. M., Tullius, T. D., and Lippard, S. J. (1981) Biochemistry 20, 3744–3748.
18. Laemmli, U. K. (1970) Nature 227, 680–685.
19. Morrissey, J. H. (1981) Anal. Biochem. 117, 307–310.
20. Chu, G., Turchi, J. J., Wahl, A. F., and Bambara, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11920–11924.
21. Suwa, A., Hirakata, M., Takeda, Y., Jesch, S. A., Mimori, T., and Hardin, J. T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6904–6908.
22. Turchi, J. J., Li, M., Blunt, T., Jego, P. A., and Jackson, S. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 320–324.
23. Boubnov, N. V., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamini, D. M., Pulaski, C. R., Band, H., Reeves, W., Hendrickson, E. A., and Weaver, D. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 890–894.
24. Rathmell, W. K., and Chu, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7623–7627.
25. Ormerod, M. G., Orr, R. M., and Peacock, J. H. (1994) Eur. J. Biochem. 211, 2123–2128.
26. Boubnov, N. V., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamini, D. M., Pulaski, C. R., Band, H., Reeves, W., Hendrickson, E. A., and Weaver, D. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 890–894.