Mechanism of DNA-Dependent Protein Kinase Inhibition by *cis*-Diamminedichloroplatinum(II)-Damaged DNA†

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ABSTRACT: We have determined the mechanism of DNA-dependent protein kinase (DNA-PK) inhibition by *cis*-diamminedichloroplatinum(II)-(cisplatin-) damaged DNA. We previously have demonstrated that Ku, the DNA binding subunit of DNA-PK, is capable of binding to DNA duplexes globally damaged with cisplatin but was unable to stimulate DNA-PK$_{cs}$, the catalytic subunit [Turchi & Henkels (1996) *J. Biol. Chem. 271*, 2992−3000]. In this report we have assessed Ku binding and DNA-PK stimulation using a series of DNA substrates containing single, site-specific d(GpG), d(ApG), and d(GpXpG) intrastrand cisplatin adducts and a substrate with a single interstrand cisplatin adduct. Results demonstrate that Ku binding is marginally decreased by the presence of cisplatin adducts on each substrate. When assayed for the ability to stimulate DNA-PK, each cisplatin-damaged substrate resulted in significantly decreased activity compared to undamaged DNA controls. The degree of inhibition of both Ku binding and kinase activity varied depending on the specific adduct employed. The inhibition of DNA-PK activity by cisplatin-damaged DNA was observed using either a synthetic peptide or human replication protein A as a substrate. Autophosphorylation of the DNA-PK$_{cs}$ and Ku subunits was also inhibited in reactions performed with cisplatin-damaged DNA, demonstrating that increased autophosphorylation of DNA-PK$_{cs}$ does not account for the decreased kinase activity observed with cisplatin-damaged DNA. Equilibrium binding and initial velocity experiments revealed a less than 2-fold increase in the K_d of Ku and the K_m of DNA-PK for DNA containing a single 1,2-d(GpG) cisplatin adduct. The mechanism of DNA-PK inhibition by cisplatin-damaged DNA can be attributed to a large decrease in the V_{max} and small increase in K_m.

DNA-dependent protein kinase (DNA-PK)[1] is a serine/threonine protein kinase that requires DNA for activity [for a review see Anderson (1993)]. DNA-PK is a heterotrimeric complex consisting of a dimeric DNA binding subunit, Ku, and a catalytic subunit, DNA-PK$_{cs}$. The gene encoding DNA-PK$_{cs}$ has been cloned and predicts a 460 kDa protein with homology to the phosphoinositol-3-kinase family, although no lipid kinase activity has been demonstrated for DNA-PK (Hartley et al., 1995). DNA structure is an important determinant in DNA-PK stimulation. Maximal stimulation of DNA-PK is observed with DNA structures for which Ku has the highest affinity (Morozov et al., 1994). While DNA-PK has been demonstrated to phosphorylate a number of proteins *in vitro* (Anderson, 1993), there is little evidence that any of these are phosphorylated *in vivo*. DNA-PK is thought to be involved in the regulation of a number of nuclear processes as the majority of proteins phosphorylated by DNA-PK are transcription factors.

DNA-PK is an essential component of the V(D)J recombination pathway and also plays a role in double-strand break repair (Jeggo et al., 1995; Errami et al., 1996; Boubnov et al., 1995). Consistent with roles in these pathways, a nonsense mutation in the DNA-PK$_{cs}$ gene has recently been identified in SCID mice (Blunt et al., 1996). These results are supported by reports of SCID lymphocytes possessing a defect in DNA-PK (Danska et al., 1996) and complementation of the SCID phenotype by expression of the DNA-PK$_{cs}$ gene (Blunt et al., 1995; Kirchgessner et al., 1995). Mutations in the 80 kDa subunit of Ku are responsible for X-ray sensitivity in xrs6 cell lines (Mizuta et al., 1996). Recently, Ku-80 knockout mice have been produced. These mice have a SCID phenotype and defects in V(D)J recombination (Zhu et al., 1996; Nussenzweig et al., 1996). Recent data have also implicated Ku in affecting nucleotide excision repair (NER) *in vitro*, although the mechanism was not identified (Calsou et al., 1996). *In vitro* and *in vivo* results support the hypothesis that cisplatin−DNA adducts are repaired via the NER (Zamble et al., 1996). Cisplatin can form a variety of DNA adducts including 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpXpG) intrastrand adducts and interstrand cross-links between two DNA strands (Fichtinger-Schepman et al., 1985). Interestingly, there are significant differences in the structures with respect to the degree of bending, unwinding, and local denaturation for each adduct (Bellon et al., 1991; Bellon & Lippard, 1990). Recently, both NMR and crystallographic data have been obtained for DNA containing a 1,2-d(GpG) cisplatin adduct and the structure has been solved (Yang et al., 1995; Takahara et

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[1] Abbreviations: DNA-PK, DNA-dependent protein kinase; cisplatin, *cis*-diamminedichloroplatinum(II); D/N, drug/nucleotide; HMG, high mobility group; DTT, dithiothreitol; bp, base pair; ss, single-stranded; RPA, replication protein A; EMSA, electrophoretic mobility shift assay; NER, nucleotide excision repair; SCID, severe combined immune deficiency.

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Table 1: DNA Substrates

DNA substrate[a]	sequence[b]	restriction enzyme[c]
12.1	5'-TCTCCTTCTT**GG**CCTCTTCCTC-3' 3'-GGAAGAA**CCGG**AGAAGGAGAGA-5'	Hae III
12.3	5'-TCTCCTTCTT**AGCT**CTTCTCTC-3' 3'-GGAAGAA**TCGA**GAAGAGAGAGA-5'	Alu I
12.5	5'-TCTCCTTCTT**GCGC**CTCTTCTC-3' 3'-GGAAGAA**CGCG**GAGAAGAGAGA-5'	Hha I
12.7	5'-GGCCTCTTCCTCTCTCCTTCTT-3' 3'- GAGAAGGAGAGAGGAAGAACAGAGA-5'	
12.9	5'-CTTCCTCTTGCTTCTCTTC-3' 3'-AGGAGAACGAAGAGAAGGA-5'	

[a] The substrates are denoted in the text based on the strand containing the cisplatin adduct. [b] The position of the cisplatin adduct is underlined. [c] The restriction enzyme recognition site is shown in boldface type.

al., 1995). How the different structures affect the activities of proteins involved in DNA metabolism remains to be determined.

We have identified DNA-PK in a screen selecting for proteins capable of binding cisplatin-damaged DNA and have demonstrated that while Ku can bind cisplatin-damaged DNA, this complex is unable to stimulate DNA-PK (Turchi & Henkels, 1996). In this report we have determined that the inhibition by cisplatin-damaged DNA is manifested via a large decrease in V_{max} and a small increase in K_m for DNA, and the degree of inhibition depends on the specific cisplatin−DNA adduct.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled nucleotides were from Pharmacia (Piscataway, NJ) and radiolabeled nucleotides were from New England Nuclear (Boston, MA). Sequenase version 2.0 was from U.S. Biochemical Corp. (Cleveland, OH). All other reagents were purchased from standard suppliers. Oligonucleotides were synthesized on a Molecular Biosystems 390 DNA synthesizer and purified by 15% polyacrylamide/7 M urea preparative DNA sequencing gel electrophoresis (Sambrook et al., 1989). The DNA oligonucleotides used in this study are presented in Table 1. Each DNA substrate was designed such that the single platination sites resided within a specific restriction enzyme cleavage site. The presence of a cisplatin adduct on the DNA renders the restriction enzyme ineffective (Ushay et al., 1981), and therefore, only DNA substrates devoid of cisplatin damage are digested.

Preparation of DNA Substrates. DNA substrates employed for EMSA were either 5′-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP or by extension with Sequenase and [α-^{32}P]dGTP following annealing to the complementary oligonucleotide. The DNA substrates were treated with cisplatin (Sigma Chemical Co.), as previously described (Turchi & Henkels, 1996), and the platinated oligonucleotides were purified by NenSorb chromatography according to the manufacturer's protocol. Platinated DNA oligonucleotides were annealed to their complementary oligonucleotide by heating the mixture to 75 °C for 5 min and allowing to cool to 25 °C over the next hour. The DNA was then extended to generate fully duplex substrates using Sequenase and dNTPs as previously described (Turchi et al., 1996). When applicable, DNA substrates were digested with the restriction enzyme noted in Table 1 and purified by 15% nondenaturing PAGE. Full-length undigested DNA substrates were excised from the gel, eluted, and precipitated with ethanol. This procedure resulted in 100% of the DNA substrates having a cisplatin adduct and assured that there was no single-strand DNA oligonucleotide present. The yield and concentration of the final product was calculated on the basis of the original specific activity of the labeled DNA, as previously described (Turchi et al., 1996). Undamaged control DNA substrates were prepared identically except cisplatin was omitted and, following annealing, the DNA duplexes were not digested with restriction enzymes prior to gel purification. The cisplatin interstrand cross-linked substrate (12.9) was prepared by annealing platinated oligonucleotide 12.9 to its complementary oligonucleotide, followed by incubation in 0.1 M $NaClO_4$ for 15 h at 37 °C. The DNA was then purified by ion-exchange chromatography using Qiagen resin (Chatsworth, CA) and purified by electrophoresis on a 10% polyacrylamide/7 M urea preparative DNA sequencing gel. The cross-linked DNA oligonucleotides migrated with an approximate molecular mass of 40−45 nucleotides and was excised from the gel, eluted, and purified as described earlier. The DNA concentration was determined by absorbance at 260 nm. DNA substrates to be used for DNA-PK assays were prepared by essentially the same procedure, except they were not labeled with [^{32}P] and, following electrophoresis, they were visualized by UV shadowing on fluorescent TLC plates. Quantification was performed by measuring the absorbance at 260 nm.

Electrophoretic Mobility Shift Assay. Mobility shift assays were performed as previously described (Turchi et al., 1996). Briefly, reactions were performed in 20 mM HEPES, pH 7.0, 1 mM DTT, 0.001% NP-40, 50 mM NaCl, 50 μg/mL bovine serum albumin, and 50 fmol of [^{32}P]DNA, incubated on ice for 30 min, and the products were separated by native polyacrylamide gel electrophoresis. Gels were dried and the products were visualized by autoradiography. Radioactivity in each band was quantified by phosphorimager analysis using ImageQuant software in volume integration mode (Molecular Dynamics, CA).

DNA-PK Assays. DNA-PK assays were performed essentially as previously described (Turchi & Henkels, 1996). Assays were performed in a final volume of 20 μL containing 20 mM HEPES, pH 7.5, 8 mM $MgCl_2$, 1 mM DTT, 5% glycerol, and [^{32}P]ATP (0.2−0.5 μCi, 125 μM) unless stated otherwise. The substrate for DNA-PK phosphorylation was a synthetic peptide (EPPLSQEAFADLWKK), based on the human p53 N-terminus, and was present in reactions at a concentration of 500 μM. Reactions were initiated at 15 s intervals, incubated at 37 °C for 15 min, terminated at 15 s intervals by the addition of an equal volume of 30% acetic acid, and spotted on Whatman P-81 filter paper. Filters were washed 5 times for 5 min each in 15% acetic acid, once briefly in 100% methanol, and dried, and radioactivity was quantified in each spot by phosphorimager analysis. Alternatively, human recombinant replication protein A (RPA) was employed as a substrate and phosphorylation was detected by autoradiography following SDS−PAGE. Reactions were terminated by the addition of SDS sample buffer and heating to 95 °C for 7 min. Samples were then separated by SDS−PAGE according to Laemmli (1970). The gels were dried and products were detected by autoradiography.

Autophosphorylation of DNA-PK subunits was performed without the addition of either RPA or peptide substrate and the ATP concentration was decreased to 25 μM. Reactions were processed as described for phosphorylation of RPA.

Protein Purification. DNA-PK was purified from HeLa cells as previously described (Turchi & Henkels, 1996). Human recombinant RPA was purified from *Escherichia coli* expressing all three subunits under the control of the T7 promoter (Henricksen et al., 1994).

RESULTS

Cisplatin−DNA Adduct Specificity of Ku Binding. We have previously demonstrated that Ku is capable of binding a 44-base duplex DNA globally damaged with cisplatin with an affinity similar to that for an undamaged control (Turchi & Henkels, 1996). This substrate was heterogenous with respect to the type of cisplatin−DNA adducts and did not allow assessment of binding to any one specific cisplatin−DNA adduct. To assess Ku binding to specific cisplatin−DNA adducts, a series of DNA substrates were designed and constructed with site-specific single cisplatin adducts. The DNA substrates employed in this study are presented in Table 1 and were constructed to assure 100% of the substrate DNA molecules had the specific cisplatin adduct and that no single-strand DNA contamination was present. The substrates were labeled with [^{32}P] and employed in an EMSA to determine Ku binding. Figure 1 shows the results of a representative EMSA using purified Ku autoantigen and substrate 12.1 without cisplatin damage (lanes 1−5), Pt-12.1 (lanes 6−10), Pt-12.3 (lanes 11−15), and Pt-12.5 (lanes 16−20). The results demonstrate that Ku is able to bind each DNA substrate. The varied intensity of the DNA substrates is a result of each substrate being labeled with [^{32}P] to differing specific activities (compare lanes 1, 6, 11, and 16). Quantification of the results is shown in panel B and reveals that Ku binds each DNA substrate with a similar affinity. EMSAs were also performed using each substrate prepared without cisplatin adducts and binding results were similar. These results are consistent with our previous results demonstrating that cisplatin adducts have little effect on DNA binding activity of Ku.

DNA-PK Activation by DNA Substrates with Specific Cisplatin−DNA Adducts. The activity of DNA-PK is dependent on double-stranded DNA, as RNA and single-stranded DNA are unable to support kinase activity (Gottlieb & Jackson, 1993; Lees-Miller et al., 1990). Our previous results demonstrated that cisplatin-damaged duplex DNA resulted in decreased kinase activity compared to undamaged DNA (Turchi & Henkels, 1996). To determine if DNA-PK inhibition is the result of a specific cisplatin−DNA adduct, each DNA substrate, platinated and unplatinated, was tested for the ability to stimulate DNA-PK activity. Figure 2 shows the results from a kinase assay and demonstrates that the presence of each cisplatin−DNA adduct significantly inhibited the ability of DNA-PK to phosphorylate the synthetic peptide. Interestingly, both the 1,2-d(GpG) and 1,2-d(ApG) adducts showed a greater degree of inhibition (79% and 98%, respectively) than the 1,3-d(GpXpG) adduct (66% inhibition) when compared to their unplatinated controls. The structural distortions induced by specific cisplatin−DNA adducts has been determined. The 1,2-d(GpG) adduct bends DNA approximately 45° toward the major groove (Takahara et al., 1995) and unwinds the DNA by 13° (Bellon et al., 1991). The 1,2-d(GpG) cisplatin adduct also mediates a transition to A-form DNA and maintains base pairing between the adducted guanine bases and their complementary cytosine bases (Takahara et al., 1995). However, the 1,3-d(GpXpG) adduct induces different structural distortions, bending by 25−35° and a greater degree of unwinding, estimated at 23° (Anin & Leng, 1990; Bellon et al., 1991). The 1,3-(GpXpG) adduct results in localized denaturation and distortion of 3−4 bases centered at the adducted site (Anin & Leng, 1990). Which of these structural distortions is responsible for decreased DNA-PK activity remains to be determined.

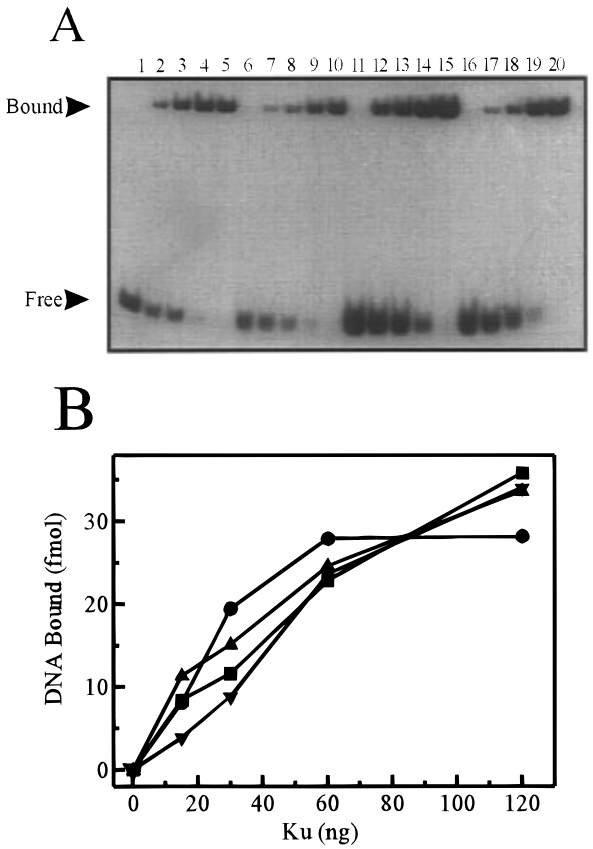

FIGURE 1: Ku binding to unplatinated or platinated DNA substrates containing internal, site-specific single cisplatin adducts. Unplatinated or platinated duplex DNA substrates were incubated with increasing concentrations of human Ku autoantigen as described in Experimental Procedures. *(*A) Lanes 1−5 are the undamaged 12.1 substrate, lanes 6−10 are the Pt-12.1 substrate, lanes 11−15 are the Pt-12.3 substrate, and lanes 16−20 are the Pt-12.5 substrate. Lanes 1, 6, 11, and 16 contain no added protein; lanes 2, 7, 12, and 17 contain 4 ng of Ku; lanes 3, 8, 13, and 18 contain 8 ng Ku; lanes 4, 9, 14, and 19 contain 16 ng of Ku; and lanes 5, 10, 15, and 20 contain 32 ng of Ku. Reaction products were separated using 12% nondenaturing PAGE and visualized by autoradiography. Arrows denote either bound or free DNA. (B) The data in panel A were quantified by phosphorimager analysis and the amount of DNA bound (femtomoles) plotted vs Ku (nanograms). Representation of DNA substrates employed in the mobility shift assay is as follows: (●), undamaged 12.1; (■), Pt-12.1; (▲), Pt-12.3, and (▼), Pt-12.5.

As Ku binds specifically to the termini of duplex DNA, a substrate was designed to contain a single cisplatin adduct at the one end of a DNA substrate. The presence of a 1,2-(GpG) adduct at the termini of a duplex DNA has been shown to induce fraying of the ends with the terminal bases being unpaired (van Boom et al., 1996). The ability of Ku to bind this DNA substrate was also unaffected by the

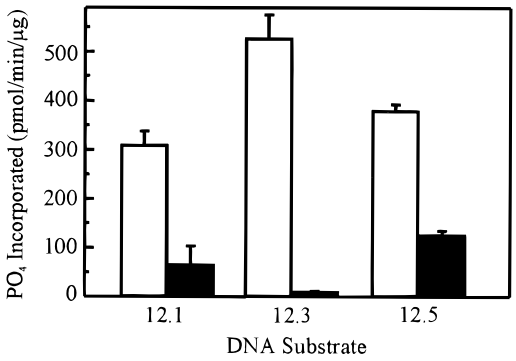

FIGURE 2: DNA-PK activation by DNA substrates containing internal, site-specific single cisplatin adducts. Reactions were performed containing 2 pmol of each DNA substrate indicated in the figure and 100 ng of DNA-PK. Phosphorylation of the synthetic peptide was determined as described in Experimental Procedures. Assays were performed in triplicate and averages ± standard deviations are presented. The open bars are undamaged and solid bars are cisplatin-damaged DNA substrates.

presence of the cisplatin adduct (Figure 3A). DNA-PK activity was still inhibited approximately 25% in reactions performed with the terminal, platinated 1,2-d(GpG) substrate (Figure 3B).

Cisplatin also forms interstrand adducts in the reaction with DNA (Fichtinger-Schepman et al., 1985). Our previous results demonstrated that the degree of inhibition of DNA-PK was dependent on the extent of DNA damage. This raised the possibility that a minor adduct could be responsible for the inhibition observed with globally damaged DNA substrates. Therefore, we prepared a DNA substrate containing a single cisplatin interstrand cross-link and assessed both Ku binding and DNA-PK activation. Similar to the other substrates tested, the presence of the interstrand cisplatin−DNA adduct had only a minor effect on Ku binding, resulting in a 50−80% decrease in binding compared to the unplatinated control DNA substrate (Figure 3A, lanes 11−20). Despite similar levels of binding, the ability to stimulate DNA was again decreased (Figure 3B). Reactions performed with the substrate containing the interstrand adduct resulted in 75% inhibition. The structure induced by a cisplatin interstrand cross-link is different than that induced by intrastrand adducts with the major difference being in the degree of unwinding of the duplex DNA, estimated at 79° (Malinge et al., 1994).

DNA-PK Phosphorylation of RPA Is Inhibited by Cisplatin-Damaged DNA. Our previous demonstration of DNA-PK inhibition by cisplatin-damaged DNA employed casein as a substrate (Turchi & Henkels, 1996). The results in Figure 2 employ a synthetic peptide substrate based on the human p53 sequence. Both of these substrates are effectively phosphorylated without being bound to DNA. Previous results have demonstrated that substrates bound to DNA are phosphorylated by DNA-PK (Gottlieb & Jackson, 1993; Anderson, 1993). RPA has been demonstrated to be phosphorylated by DNA-PK *in vitro* (Brush et al., 1994). However, phosphorylation has no effect on DNA replication or NER (Pan et al., 1995). Therefore, RPA was purified from *E. coli* overexpressing all three subunits as previously described (Henricksen et al., 1994) and was employed as a substrate for DNA-PK in reactions containing platinated and unplatinated DNA. DNA-PK assays were performed and products were separated by SDS−PAGE and detected by

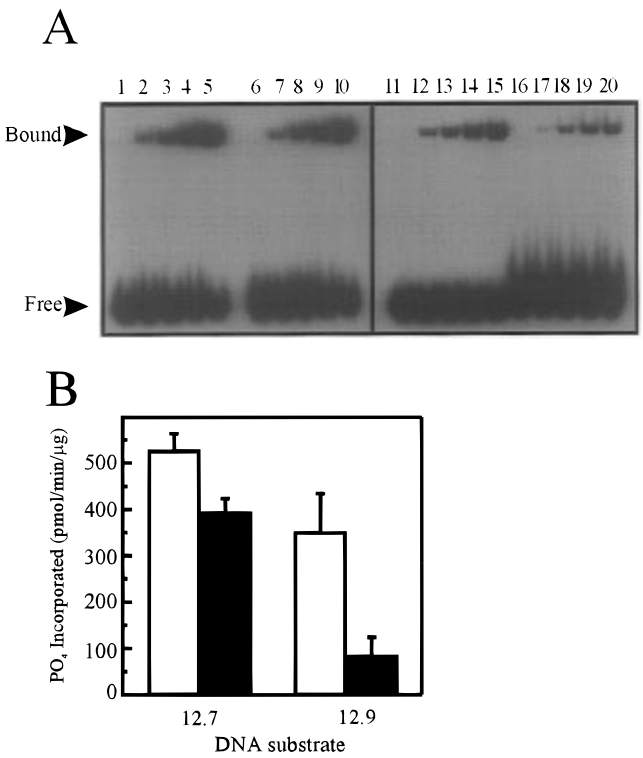

FIGURE 3: Ku binding and DNA-PK activation by terminal intrastrand and interstrand cisplatin−DNA adducts. (A) The 1,2d(GpG) terminal and interstrand cisplatin adduct DNA substrates were prepared according to Experimental Procedures. Undamaged 12.7 substrate (lanes 1−5), Pt12.7 (lanes 6−10), undamaged 12.9 (lanes 11−15), and the interstrand cross-linked 12.9 substrate (lanes 16−20) were assessed in EMSA with purified Ku. Ku was omitted from the reactions (lanes 1, 6, 11, and 16) or was present at 15 ng (lanes 2, 7, 12, and 17), 30 ng (lanes 3, 8, 13, and 18), 60 ng (lanes 4, 9, 14, and 19), and 120 ng of Ku (lanes 5, 10, 15, and 20). Reaction products were separated by 10% nondenaturing PAGE and visualized by autoradiography. Bound and free DNA are indicated by the arrows. (B) DNA-PK assays were performed using unplatinated (open bar) and platinated (solid bar) substrate 12.7 and 12.9, as described in the legend to Figure 1. Results are presented as the average ± standard deviation of triplicate determinations.

autoradiography. The results shown in Figure 4 demonstrate that the 32 kDa subunit of RPA is phosphorylated dependent upon DNA. Lane 5, a reaction performed without DNA, shows no phosphorylation, while reactions performed with unplatinated (lane 6) or platinated (lane 7) substrate 12.1 revealed phosphorylation of the 32 kDa subunit of RPA. The level of phosphorylation in the reaction containing platinated DNA is approximately 40% of that observed in the reaction with unplatinated DNA. These results showing inhibition of DNA-PK phosphorylation by cisplatin-damaged DNA are consistent with our previously published data and those presented in Figure 2. A positive control reaction was performed using activated calf thymus DNA as a substrate (lane 8). Activated calf thymus DNA contains a variety of structures including single-stranded segments, nicks, and gaps with which RPA and DNA-PK can bind. Use of this substrate shows a greater degree of DNA-PK activation and phosphorylation of RPA. The decreased phosphorylation in reactions performed with the synthetic oligonucleotides is a result of the lower DNA concentrations employed in the reactions. The activity of DNA-PK increased linearly with substrate 12.1 up to a level of 5 pmol of DNA/reaction using RPA as a substrate (data not shown). This DNA concentra-

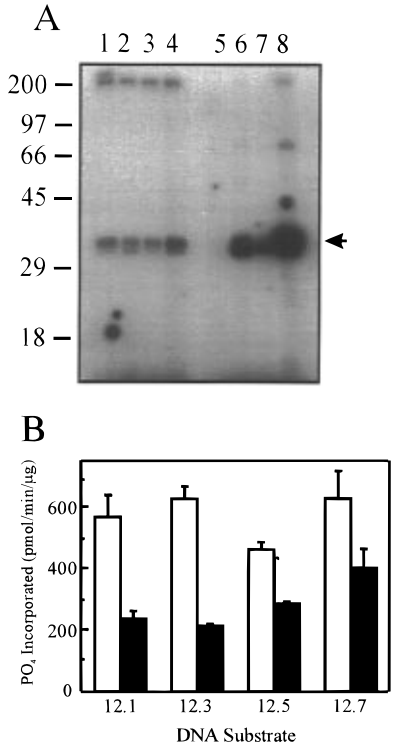

FIGURE 4: DNA-PK-dependent phosphorylation of RPA is inhibited by cisplatin-damaged DNA. (A) Human recombinant RPA (544 ng) was incubated in kinase reactions as described in Experimental Procedures. Reactions were terminated, and products were separated by SDS−12% PAGE and visualized by autoradiography. Lanes 1−4 contain 1.5 μg of ssM13mp18 DNA; lanes 1 and 5, no duplex DNA; lanes 2 and 6, substrate 12.1; lanes 3 and 7, Pt-12.1 substrate; and lanes 4 and 8, 100 ng of activated calf thymus DNA. The positions of molecular weight markers are shown on the left axis and the arrow denotes the 32 kDa subunit of RPA. (B) Reactions were performed in triplicate using the DNA substrate noted in the figure. RPA was increased to 1.3 μg/assay and ssM13mp18 DNA was omitted. Quantification of kinase activity was performed by phosphorimager analysis of the 32 kDa RNA band and is presented as the average ± standard deviation.

tion is considerably greater than that required to bind all the Ku in the reaction, suggesting that the association of the DNA-PK$_{cs}$ subunit may be the limiting step in the reaction. The decrease in RPA phosphorylation by DNA-PK observed with cisplatin-damaged DNA substrate 12.1 (lane 7) could be the result of inefficient binding of RPA to that substrate. The ability of RPA to bind substrate 12.1 was assessed in an EMSA and did demonstrate that RPA binds both the platinated and unplatinated DNA substrate. Interestingly, RPA bound the platinated substrate with a higher affinity than the undamaged control (manuscript in preparation). Control reactions were also performed and are shown in lanes 1−4, which are identical to lanes 5−8 except that ssM13mp18 DNA was added to the reactions to provide RPA binding sites. The activity of DNA-PK is inhibited by the ss-DNA, but even under these conditions a slight decrease in RPA phosphorylation was observed. The results of DNA-PK phosphorylation of RPA using the various DNA substrates are shown in Figure 4B. The phosphorylation of RPA closely follows that of the synthetic peptide, with the greatest degree of inhibition observed with the 1,2-d(GpG) and 1,2-d(ApG) adducts, 60% and 66%, respectively. The 1,3-d(GpXpG) adduct resulted in less inhibition (38%) and the terminal 1,2-d(GpG) adduct resulted in the least inhibition (36%). The degree of inhibition of RPA phosphorylation is

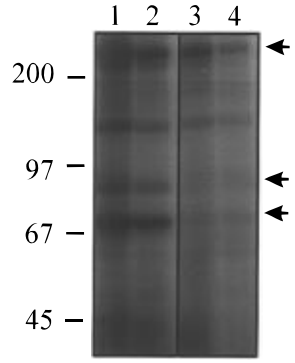

FIGURE 5: Autophosphorylation of DNA-PK using undamaged and cisplatin-damaged DNA substrates. Autophosphorylation reactions were performed as described in Experimental Procedures except the ATP concentration was decreased to 20 μM and reactions were incubated for 30 min. Reactions were performed without the addition of synthetic peptide (lanes 1 and 2) or with peptide added to a final concentration of 0.5 mM (lanes 3 and 4). Reactions were performed using undamaged substrate 12.1 (lanes 1 and 3) or platinated 12.1 (lanes 2 and 4). Following incubation, products were separated by SDS−8% PAGE, dried, and visualized by autoradiography. The positions of molecular weight markers are shown on the left axis and the arrows denote the positions of the 460 kDa DNA-PK$_{cs}$ and the Ku-80 and Ku-70 subunits that are phosphorylated.

similar to that observed for the synthetic peptide (Figure 2). These results suggest that binding of the substrate being phosphorylated is unaltered in reactions performed with cisplatin-damaged DNA.

Autophosphorylation of DNA-PK$_{cs}$. Recently, the activity of DNA-PK has been demonstrated to be modulated by the autophosphorylation of the catalytic subunit (Chan & Lees-Miller, 1996). DNA-PK can autophosphorylate both Ku subunits with no effect on kinase activity, but phosphorylation of the catalytic subunit can decrease kinase activity (Lees-Miller et al., 1990; Chan & Lees-Miller, 1996). Therefore, one mechanism of inhibition of DNA-PK could be increased autophosphorylation of the catalytic subunit in reactions performed with cisplatin-damaged DNA compared to an undamaged DNA control. The degree of autophosphorylation was assessed in a series of reactions containing platinated or unplatinated DNA without the addition of peptide substrate or RPA. The results shown in Figure 5 demonstrate that autophosphorylation of both Ku subunits and DNA-PK$_{cs}$ is slightly decreased in reactions performed with cisplatin-damaged DNA (lane 2) compared to undamaged DNA (lane 1). The addition of synthetic peptide to the autophosphorylation reactions resulted in a decrease in phosphorylation of each DNA-PK subunit, consistent with previous results (Chan & Lees-Miller, 1996).

Kinetic Analysis of DNA-PK. The mechanism by which cisplatin-damaged DNA decreases the phosphorylation activity of DNA-PK was assessed in initial velocity studies and equilibrium binding assays. The ability of Ku to bind DNA substrate 12.1, platinated and unplatinated, was assessed in equilibrium binding assays. The concentration of DNA was varied and K_d was determined by scatchard analysis. The results are presented in Figure 6A and an increase of 40% in the K_d for DNA was observed when platinated DNA was employed. This difference is unable to fully account for the 75−80% decrease in DNA-PK activity observed with cisplatin-damaged DNA. Therefore, initial velocity measurements were performed using DNA as the variable substrate

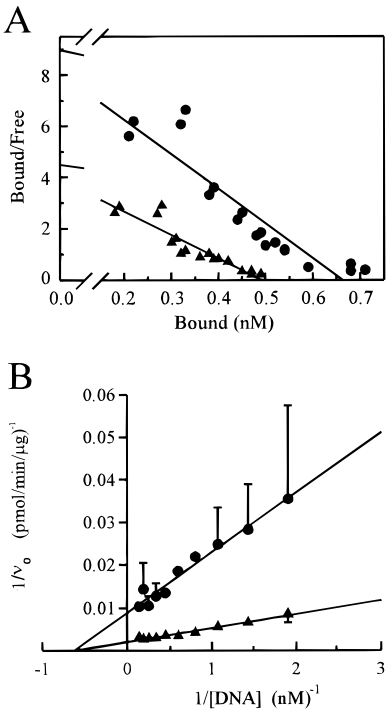

FIGURE 6: Equilibrium binding and initial velocity analysis of DNA-PK interaction with undamaged and cisplatin-damaged DNA. (A) EMSAs were performed as described in Experimental Procedures. Ku (100 ng) was incubated with varying concentrations of either undamaged (▲) or cisplatin-damaged (●) substrate 12.1. Equilibrium was reached in 30 min and products were separated by native gel electrophoresis. Quantification of both bound and free DNA was performed by phosphorimager analysis and *B/F* was plotted versus DNA bound. Each reaction was performed in duplicate and all data points are shown. Linear regression analysis was performed and the dissociation constant was determined by the slope of the line. The regression coefficients were 0.84 and 0.86 for the undamaged and damaged DNA, respectively. (B) Phosphorylation of the synthetic peptide was determined in reactions containing 100 ng of DNA-PK and varying concentrations of DNA substrate 12.1 (▲) or Pt12.1(●). Reactions were performed in duplicate and the averages ± standard deviations are presented in double-reciprocal plots. Kinetic constants were determined from linear regression analysis of the data and the regression coefficients were 0.91 and 0.79 for the undamaged and cisplatin-damaged DNA, respectively.

Table 2: Kinetic Constants

DNA	K_d[a] (nM)	K_m (DNA) (nM)	V_{max} (pmol min^{-1} μg^{-1})	k_{cat}/K_m (nM·min)$^{-1}$
12.1	0.074	79.6	490	3.75
Pt12.1	0.110	130	142.5	0.668
12.5	ND[a]	143	555	2.35
Pt12.5	ND	154	290	1.15
12.7	ND	49.2	574	7.11
Pt12.7	ND	69.9	500	4.37
12.9	ND	59.0	789	8.15
Pt12.9	ND	119	437	2.24

[a] K_d values were calculated from Scatchard plots and represent the dissociation constant for Ku binding to DNA in the absence of DNA-PK$_{cs}$. [b] ND, not determined.

and kinetic constants were determined via double-reciprocal plots (Figure 6B). The results, summarized in Table 2, demonstrate that the K_m for platinated DNA is 67% greater than that for undamaged. The results are consistent with the K_d measurements of Ku binding unplatinated DNA. The K_m value obtained in these experiments represents the association of DNA-PK$_{cs}$ with DNA. Since the ability of DNA-PK$_{cs}$ to associate with DNA is via the Ku component, the results suggest that the DNA-PK$_{cs}$ joining the Ku−DNA complex is limiting. However, the ability of DNA-PK$_{cs}$ to form the active trimeric complex, joining the Ku−DNA complex, is independent of whether the DNA contains a cisplatin adduct. The initial velocity studies demonstrated a significant decrease in V_{max} (from 490 to 142.5 pmol min^{-1} μg^{-1}) when the reactions were performed with cisplatin-damaged DNA. This difference combined with the increase in K_m yields a 5-fold greater specificity constant for undamaged DNA and accounts for the decreased phosphorylation in reactions containing cisplatin-damaged DNA. The same general trend was observed for each of the other intrastrand cisplatin−DNA adducts. The decrease in activity observed with cisplatin could largely be attributed to decreased maximum velocities. Kinetic constants obtained using the interstrand DNA substrate yielded a greater increase in K_m, nearly 2-fold, compared to the intrastrand adducts. This result is consistent with the Ku binding data presented in Figure 3. There was also a decrease in V_{max} in reactions performed with the interstrand cross-linked substrate, resulting in a decrease in the specificity constant by 70%. These results demonstrate that binding to the interstrand substrate plays a greater role in cisplatin inhibition of DNA-PK compared to the intrastrand-damaged DNA substrates.

DISCUSSION

Chemotherapeutic treatment of ovarian cancer with cisplatin is often initially effective; however, recurrence and resistance is a clinical problem (Ozols, 1995). The efficacy of cisplatin is the result of DNA adduct formation and the structure of a number of these adducts has been determined, including the major 1,2-d(GpG)cisplatin−DNA adduct (Takahara et al., 1995). We have been studying how mammalian proteins with an affinity for cisplatin−DNA adducts interact with and alter the metabolism of the adducts, as well as how the adducted DNA influences the activity of the specific proteins (Turchi & Henkels, 1996; Turchi et al., 1996). The Ku autoantigen is a fairly abundant nuclear protein that is involved in V(D)J recombination and double-strand break repair and in the DNA binding subunit of the human DNA-PK (Jeggo et al., 1995). DNA-PK *in vitro* catalyzes the phosphorylation of a number of transcription factors and may be involved in the regulation of DNA metabolism (Anderson, 1993).

We have identified DNA-PK as a protein that binds to DNA damaged with cisplatin (Turchi & Henkels, 1996) and demonstrated that while Ku affinity for damaged DNA is only minimally decreased, DNA-PK activity is dramatically inhibited. Interestingly, while all the cisplatin−DNA adducts tested inhibited DNA-PK activity, the degree of inhibition varied depending on the specific adduct (Figures 2 and 4B). These results suggest that the different DNA structures induced by the cisplatin adducts interact with DNA-PK differently. The DNA binding component of DNA-PK, Ku, shows essentially no differential binding to any of the cisplatin−DNA adducts, suggesting that the difference must lie in the interaction with the DNA-PK$_{cs}$ subunit. This is supported by the initial velocity studies demonstrating a decreased V_{max}, which accounts for the majority of the inhibition by cisplatin-damaged DNA (Table 2). Results obtained with a cisplatin adduct at the end of a DNA substrate show less of an inhibitory effect on DNA-PK. A

terminal cisplatin−DNA adduct induces fraying at the end with the adducted GpG sequence not pairing with their complementary bases (Takahara et al., 1995). The increased activity observed with these substrates compared to DNA substrates with cisplatin adducts at internal positions suggests that the platinum itself is not responsible for the altered activity, but the DNA structure induced by the cisplatin is responsible for the decreased activity. In this respect, cisplatin-damaged DNA may serve as a probe for DNA-PK active-site conformation to help elucidate how DNA is responsible for activation of kinase activity. The hypothesis that the DNA structure is important for DNA-PK activation is consistent with previous results demonstrating that DNA-PK_{cs} can be cross-linked to DNA (Gottlieb & Jackson, 1993; Lees-Miller et al., 1990). In addition, single-strand DNA can bind Ku but is unable to stimulate DNA-PK, demonstrating that there is more to DNA-PK activation than having Ku bound to DNA (Gottlieb & Jackson, 1993; Lees-Miller et al., 1990). DNA-PK is thought to form the heterotrimeric complex on DNA and may not associate independent of DNA (Suwa et al., 1994). Our results are consistent with this conclusion as the K_d for Ku binding to DNA is in the subnanomolar range while the K_m for DNA-PK binding to DNA is in the 100 nM range (Table 2). These results demonstrate that the first step in the activation of DNA-PK is Ku binding to DNA, followed by the addition of DNA-PK_{cs}. In reactions performed with cisplatin-damaged DNA, binding of Ku is only moderately decreased and the association of DNA-PK_{cs} is also moderately affected. The assembly of Ku on double-strand DNA has been studied and the p70 subunit displayed an affinity for duplex DNA similar to that for the p70/80 heterodimer (Wang et al., 1994a). In addition, free p70 *in vivo* was found associated with chromatin in the absence of p80 subunit (Wang et al., 1994b). More recent evidence has demonstrated that both subunits are necessary for binding DNA and has identified the regions of each protein required for interaction (Wu & Lieber, 1996). Our results have measured the affinity of the heterodimer for DNA and the ability to stimulate DNA-PK_{cs}, and the possible role of p70 monomer binding cisplatin-damaged DNA remains to be addressed.

The association of DNA-PK with damaged DNA and a decrease in activity as a result could alter the metabolism of the specific cisplatin adducts. DNA-PK *in vitro* phosphorylates RPA, the human single-stranded DNA binding protein, which is involved in both DNA replication and repair (Wold, 1997). RPA is also phosphorylated *in vivo* in response to UV-induced DNA damage (Carty et al., 1994). However, DNA-PK phosphorylation of RPA does not alter its ability to participate in DNA replication or NER *in vitro* (Pan et al., 1995). The DNA-PK dependent phosphorylation of RPA has recently been implicated in regulating DNA replication *in vitro* via altering how DNA-PK interacts and possibly phosphorylates other proteins involved in DNA replication (Henricksen et al., 1996). How this activity is altered by cisplatin-damaged DNA and the effect on DNA replication and repair is an area that needs to be addressed.

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