Human Replication Protein A Preferentially Binds Cisplatin-Damaged Duplex DNA in Vitro†

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ABSTRACT: Fractionation of human cell extracts by cisplatin−DNA affinity chromatography was employed to identify proteins capable of binding cisplatin-damaged DNA. A specific protein−DNA complex, termed DRP-3, was identified in an electrophoretic mobility shift assay (EMSA) using a cisplatin-damaged DNA probe. Using this assay we purified DRP-3 and the final fraction contained proteins of 70, 53, 46, 32, and 14 kDa. On the basis of subunit molecular weights, antibody reactivity, and DNA binding activities, DRP-3 was identified as human replication protein A (hRPA). Therefore, we assessed the binding of recombinant human RPA (rhRPA) to duplex cisplatin-damaged DNA in vitro. Global treatment of a highly purified completely duplex 44-bp DNA with cisplatin resulted in a 10−20-fold increase in rhRPA binding compared to the undamaged control. The stability of the RPA−DNA complexes was assessed, and NaCl and $MgCl_2$ concentrations that completely inhibited rhRPA binding to undamaged DNA had only a minimal effect on binding to duplex platinated DNA. We assessed rhRPA binding to a duplex DNA containing a single site-specific 1,2-d(GpG) cisplatin adduct, and the results revealed a 4−6-fold increase in binding to this DNA substrate compared to an undamaged control DNA of identical sequence. These results are consistent with RPA being involved in the initial recognition of cisplatin-damaged DNA, possibly mediating DNA repair events. Therefore, we assessed how another cisplatin DNA binding protein, HMG-1, affected the ability of rhRPA to bind damaged DNA. Competition binding assays show minimal dissociation of either protein from cisplatin-damaged DNA during the course of the reaction. Simultaneous addition experiments revealed that HMG-1 binding to cisplatin-damaged DNA was minimally affected by rhRPA, while HMG-1 inhibited the damaged-DNA binding activity of rhRPA. These data are consistent with HMG-1 blocking DNA repair and possibly having the capability to enhance the cytotoxic efficacy of the drug cisplatin.

The ability of mammalian cells to repair bulky adduct damage to DNA depends on the ability to recognize the damaged DNA in a background of vast amounts of undamaged DNA. To accomplish this task, the nucleotide excision repair (NER)[1] pathway depends on proteins that display a higher affinity for damaged DNA and coupling repair with transcription (reviewed in ref *1*). The development of an in vitro NER assay (*2*) and the use of xeroderma pigmentosum (XP) cells has enabled the identification and characterization of mammalian proteins required for NER. The entire NER pathway has now been reconstituted with purified proteins (*3*, *4*). The initial event in NER involves recognition of the damaged DNA mediated via XPA and RPA (*5*). The XPA protein has been purified, cloned, and extensively characterized (*6*, *7*). This 31 kDa protein displays a preferential binding to DNA damaged with a variety of agents, including UV-induced damage, cisplatin−DNA adducts, and acetylaminofluorine−DNA adducts (*7*, *8*). XPA and RPA have been demonstrated to interact in the absence of DNA, suggesting that this complex may mediate the initial recognition of damaged DNA (*9*, *10*). Interestingly, recent results have demonstrated that RPA can bind directly to UV-induced DNA damage in the absence of XPA (*11*). RPA displayed a higher affinity for UV-damaged duplex DNA in reactions performed in the presence of $MgCl_2$, and it was suggested that single-stranded character in the UV-damaged DNA may mediate the increased affinity (*11*). RPA has also been shown to bind DNA damaged with cisplatin (*12*), suggesting that it may be involved in repair of various types of DNA damage.

In addition to the XPA and RPA proteins, there are numerous other nuclear proteins that are not part of the NER pathway that are able to bind cisplatin-damaged DNA. This set of proteins, including the HMG domain proteins, may play a role in the metabolism of cisplatin−DNA adducts and possibly affect the cytotoxic efficacy of the drug. One of the cellular mechanisms of cisplatin resistance is enhanced DNA repair (*13*, *14*). These damage recognition proteins, DRPs, could bind the damaged DNA and stimulate or inhibit the repair process. HMG-1, the prototypical HMG domain

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[1] Abbreviations: HMG, high mobility group; rhRPA, recombinant human replication protein A; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; DRP, damage recognition proteins; D/N, drug to nucleotide; NER, nucleotide excision repair; XP, xeroderma pigmentosum; bp, base pairs.

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protein, has been purified and characterized with respect to binding cisplatin-damaged DNA (*15*−*17*). HMG-1 has also been implicated in inhibiting DNA repair and potentiating the cytotoxic action of cisplatin (*18*).

Here we have purified a protein that binds cisplatin-damaged DNA, DRP-3. Comparison of the protein subunits, the complexes formed with DNA, and EMSA supershift analysis confirms that DRP-3 is RPA. We have demonstrated by EMSA analysis that recombinant human RPA, rhRPA, preferentially binds duplex cisplatin-damaged DNA compared to the undamaged control DNA dependent upon salt concentration. There is also preferential binding to a duplex substrate containing a single 1,2-d(GpG) cisplatin−DNA adduct. Competition EMSA analysis reveals that HMG-1 can compete for RPA binding to a cisplatin-damaged DNA. These results are discussed with respect to cisplatin−DNA binding and the repair of cisplatin−DNA adducts.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled nucleotides were from Pharmacia (Piscataway, NJ) and radiolabeled nucleotides were from New England Nuclear (Boston, MA). Oligonucleotides were synthesized on a Molecular Biosystems 390 DNA synthesizer and purified by 15% polyacrylamide/7 M urea preparative DNA sequencing gel electrophoresis. T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Sequenase (version 2.0) was from U.S. Biochemical Corp. (Cleveland, OH). Cisplatin was from Sigma (St. Louis, MO). All other reagents were purchased from standard suppliers.

DNA Substrates. The DNA substrates employed for EMSA were either 5′-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP or following annealing to the complementary oligonucleotide labeled by extension with Sequenase, [α-^{32}P]-dGTP, and dNTPs. The duplex 44-bp DNA and duplex 25-bp DNA substrates were prepared as previously described (*17*, *19*). The 44-bp DNA was treated with mung bean nuclease following native gel purification to ensure the substrate was completely duplex. The single-stranded 24-base DNA was prepared as previously described (*20*).

DNA Platination. DNA oligonucleotides or substrates were incubated with varying concentrations of cisplatin in 1 mM $NaHPO_4$ (pH 7.5) and 3 mM NaCl for 16 h at 37 °C in the dark. The duplex 44-bp DNA was globally platinated at a D/N ratio of 0.2:1. The duplex 25-bp DNA was platinated and prepared as described to ensure that 100% of the DNA substrates had a single 1,2-d(GpG) cisplatin−DNA adduct (*19*). The DNA was purified from unreacted cisplatin by G-50 spin column chromatography (*21*). The counts per minute recovered was determined by liquid scintillation counting of an aliquot of the eluate, and on the basis of the specific activity obtained in the original labeling reactions, the picomoles of DNA recovered was calculated.

Electrophoretic Mobility Shift Assay. EMSAs were performed as previously described with the following modifications (*17*). Assays were performed in 20 μL reactions containing 20 mM HEPES (pH 7.0), 1 mM DTT, 0.001% Nonidet P-40, 50 μg/mL bovine serum albumin, and 50 fmol of [^{32}P]DNA, unless stated otherwise, and equilibrium was achieved by incubation at room temperature for 30 min (data not shown). For the competition EMSA, either HMG-1 or rhRPA was preincubated with the DNA substrate for 30 min, and then competitor protein was added for an additional 30 min, or a mixture of both proteins was incubated with the DNA for 30 min. Reaction products were separated by either 4% or 6% native polyacrylamide gel electrophoresis (PAGE). Gels were dried, and products were visualized by autoradiography and quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Protein Purification. All procedures were performed at 4 °C and all buffers were supplemented with 1 mM DTT, 0.5 mM PMSF, 0.5 μg/mL leupeptin and pepstatin A, and 1 mM EDTA. DRP-3 was purified from HeLa whole cell extracts using a cisplatin−DNA−Sepharose affinity column as previously described (*17*, *22*). The cisplatin−DNA−Sepharose pool was diluted to a conductivity equal to 0.1 M NaCl and applied to a 30 mL Affi-Gel blue column equilibrated in 50 mM Tris-HCl, pH 7.5, 20% glycerol, and 100 mM NaCl. Bound protein was eluted sequentially from the column with 100 mL each of 0.5 M KCl, 0.5 M NaSCN, and 1.5 M NaSCN. The 1.5 M NaSCN pool was dialyzed versus 20 mM KP_i, pH 7.0, and 20% glycerol and applied to a 10 mL hydroxylapatite column equilibrated in the same buffer. The column was washed and bound protein was eluted with a linear gradient from 20 mM to 0.4 M KP_i in 20% glycerol. The peak of DRP-3 binding activity was pooled, diluted, and further purified on a 5 mL Q-Sepharose column. Protein was eluted from the Q-Sepharose column with a linear gradient from 0.1 M to 1 M NaCl in 50 mM Tris-HCl, pH 7.5, and 20% glycerol. Fractions containing DRP-3 binding activity were pooled and frozen at −85 °C. Recombinant human RPA (rhRPA) was purified from *Escherichia coli* expressing all three subunits under the control of the T7 promoter as previously described (*23*). HMG-1 was purified to homogeneity from calf thymus as previously described (*17*).

RESULTS

Purification and Identification of DRP-3. We have previously purified two proteins from HeLa whole cell extracts on the basis of their retention on a cisplatin−DNA−Sepharose column, HMG-1 and DNA-PK/Ku (*17*, *22*). Here we show the purification of a third damage recognition protein, DRP-3, and its characterization with respect to binding duplex cisplatin-damaged DNA. Initial fractionation of cellular extracts was accomplished on a cisplatin−DNA−Sepharose column as described under Experimental Procedures. Further purification was achieved with Affi-Gel blue, hydroxylapatite, and Q-Sepharose columns (see Experimental Procedures). SDS−PAGE analysis of the of DRP-3 column pools is shown in Figure 1A. Protein subunits of 70, 53, 46, 32, and 14 kDa are concentrated in the DRP-3 column pools (lanes 1−5) as indicated by the arrows, and these protein subunits copurified with the cisplatin−DNA binding activity (data not shown). This protein composition is similar to that of human replication protein A, hRPA (reviewed in ref *24*). In addition, hRPA, which is involved in nucleotide excision repair, has been shown to bind DNA damaged with cisplatin (*12*). To confirm the hypothesis that DRP-3 is hRPA, EMSA analysis using a single-stranded 24-base DNA was performed to compare the DRP-3−DNA complex to that formed using a highly purified recombinant human RPA preparation (Figure 1B). Lane 1 represents a control without added protein, while lanes 2 and 3 are from reactions

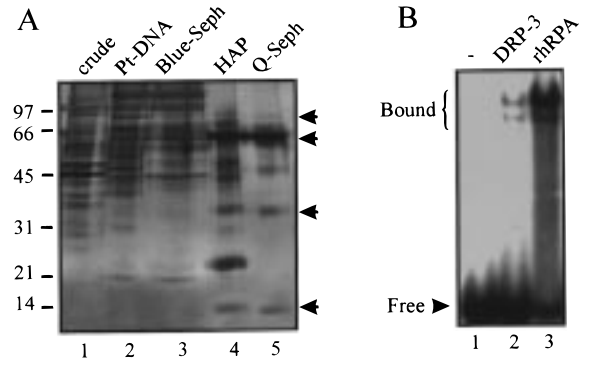

FIGURE 1: Identification of DRP-3 as hRPA. (A) SDS−PAGE analysis of hRPA fractions. Each hRPA fraction (3 μg) was analyzed on a 10−20% gradient polyacrylamide SDS gel and stained with silver as described under Experimental Procedures. Lane 1, crude extract; lane 2, cisplatin−DNA−Sepharose; lane 3, blue Sepharose; lane 4, hydroxylapatite; lane 5, Q-Sepharose. The position of molecular mass markers and mass in kilodaltons is indicated on the left axis. The arrows indicate the RPA subunits. (B) EMSA analysis of DRP-3 and rhRPA binding an undamaged ^{32}P-labeled single-stranded 24-base DNA. Lane 1, without added protein; lane 2, 40 ng of DRP-3; lane 3, 10 ng of rhRPA. The position of free and bound DNA is indicated. The products were separated on a 6% native polyacrylamide gel and visualized by autoradiography.

containing DRP-3 and rhRPA, respectively. DRP-3 and rhRPA result in the formation of a DNA−protein complex with the same mobility, suggesting that DRP-3 is RPA. The faster migrating protein−DNA complex shown in lanes 2 and 3 could be the result of proteolytic cleavage of the 70 kDa subunit to 53 kDa, which would affect migration in a native gel. Confirmation of the identity of DRP-3 was obtained by supershift analysis of the DRP-3−DNA complex using a monoclonal antibody to the 32 kDa subunit of hRPA (data not shown). Consistent with DRP-3 being RPA, a significantly greater affinity for single-stranded DNA compared to duplex DNA was observed (data not shown).

Preferential Binding of rhRPA to Duplex Cisplatin-Damaged DNA. RPA has been characterized with respect to DNA binding specificity on single-stranded and double-stranded DNA, as well as binding UV-damaged DNA (reviewed in ref *24*). In addition, a cisplatin−DNA−protein complex observed in an EMSA with whole cell extracts was attributed to RPA (*12*). Here we have assessed the characteristics of highly purified recombinant RPA binding to either undamaged or globally cisplatin-damaged duplex 44-bp DNA (Figure 2A). In reactions containing 50 mM NaCl, similar binding of rhRPA was observed to undamaged and cisplatin-damaged duplex 44-bp DNA in the absence of $MgCl_2$ (lanes 1 and 2). Preferential binding of rhRPA to the damaged substrate was observed in reactions that were supplemented with 2 mM $MgCl_2$ (lanes 3 and 4). Considering the RPA has a much greater affinity for single-stranded DNA, it was imperative to ensure that no single-stranded DNA was contaminating the DNA substrate preparations. Therefore, purification on high-percentage polyacrylamide gels and treatment with mung bean nuclease were employed to remove any single-stranded DNA present in our substrate preparations. EMSA analyses of rhRPA binding to the purified mung bean nuclease-treated DNA substrates showed that, even in the absence of $MgCl_2$, binding to the undamaged duplex DNA substrate is abolished while binding to the cisplatin-treated DNA is still maintained (lanes 5 and 6). The addition of $MgCl_2$ to the reactions had no effect on the already low binding to the undamaged DNA and a minimal decrease in binding to the cisplatin-damaged DNA substrate (lanes 7 and 8). This suggests that single-stranded DNA contamination was responsible for rhRPA binding to the undamaged DNA and $MgCl_2$ serves to decrease the nonspecific binding to the undamaged DNA. Quantification of the results reveals greater than 10-fold enhanced binding of rhRPA to the duplex damaged DNA (Figure 2B). This ability to recognize damaged DNA preferentially is consistent with a role for RPA in the initial steps of NER (*5*). All subsequent experiments with the 44-bp DNA substrate were conducted with the gel-purified and mung bean nuclease-treated DNAs to ensure that no single-stranded DNA was present.

Effect of Salt on rhRPA Binding Undamaged or Cisplatin-Damaged Duplex DNA. To determine the maximum degree of damaged DNA discrimination, experiments were performed titrating either NaCl or $MgCl_2$ into the reactions. EMSA analysis of rhRPA binding to undamaged or cisplatin-damaged duplex 44-bp DNA with varying NaCl concentrations is shown in Figure 3A. Lanes 1−10 represent the undamaged control DNA, and lanes 11−20 represent the cisplatin-treated DNA. The slower migration of the free cisplatin-treated DNA compared to the untreated DNA is denoted by the arrowhead and is the result of the cisplatin−DNA adducts, which bend and distort the DNA, altering its migration in the gel (*25*). The control reactions without added rhRPA for the undamaged and cisplatin-damaged DNA are shown in lanes 1 and 11, respectively. In the absence of any NaCl, rhRPA binds both the undamaged and cisplatin-treated substrates similarly (lanes 2 and 12, respectively). With increasing NaCl, rhRPA binding to undamaged DNA decreases dramatically, while binding to the cisplatin-treated DNA is minimally affected. The addition of NaCl to a concentration of 50 mM resulted in complete inhibition of binding the undamaged DNA, consistent with the results presented in Figure 2. The titration of $MgCl_2$ into reactions in the absence of NaCl (Figure 4A) also abolishes rhRPA binding to the undamaged substrate (lanes 1−10), while binding to the damaged DNA (lanes 11−20) is still maintained. These results demonstrate that the complex between RPA and cisplatin-damaged DNA is less sensitive to ionic strength and divalent cations and, therefore, is more stable when compared to the complex formed with undamaged DNA. In addition, the salt may serve to stabilize the undamaged DNA-duplex structure, whereas the damaged DNA structure is minimally stabilized and therefore still supports rhRPA binding.

Concentration Dependence of rhRPA Binding Duplex Damaged DNA. Under the conditions determined to minimize rhRPA binding to undamaged DNA, 50 mM NaCl and 2 mM $MgCl_2$, increasing concentrations of rhRPA were employed in an EMSA using undamaged control DNA or cisplatin-damaged 44-bp duplex DNA (Figure 5A). The presence of both NaCl and $MgCl_2$ abolishes almost all rhRPA binding to undamaged DNA and results in the highest level of preferential binding to cisplatin-damaged DNA. The control reactions without added rhRPA for the undamaged and cisplatin-damaged DNA are shown in lanes 1 and 6, respectively. Lanes 1−5 represent the undamaged duplex

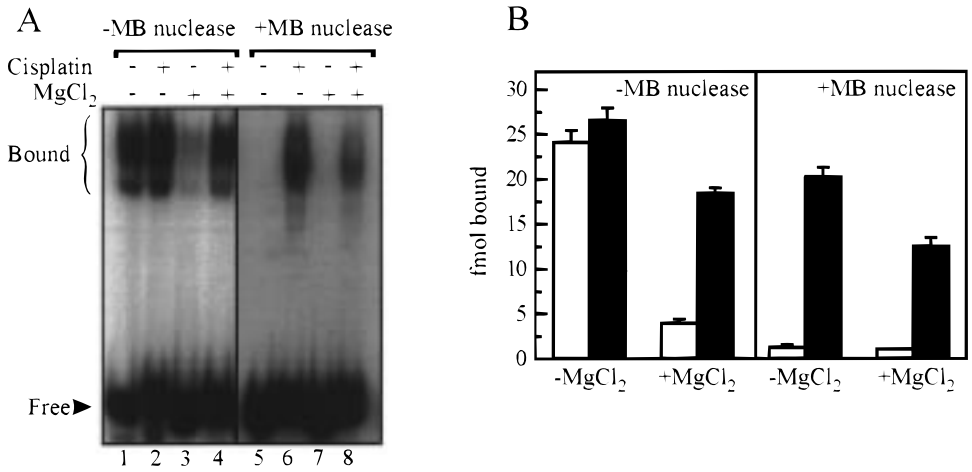

FIGURE 2: RPA cisplatin-damaged DNA discrimination is enhanced by isolation of completely duplex DNA. The 44-bp fully duplex DNA was prepared as described in Experimental Procedures. (A) EMSAs were performed with 50 fmol of DNA, 50 ng (425 fmol) of rhRPA, and buffer containing 50 mM NaCl in the absence or presence of 2 mM $MgCl_2$ as indicated. The 44-bp DNA was left untreated (lanes 1−4) or treated with mung bean nuclease (lanes 5−8). Lanes 1 and 5, undamaged duplex DNA in the absence of $MgCl_2$; lanes 2 and 6, cisplatin-damaged duplex DNA in the absence of $MgCl_2$; lanes 3 and 7, undamaged duplex DNA in the presence of $MgCl_2$; lanes 4 and 8, cisplatin-damaged duplex DNA in the presence of $MgCl_2$. The positions of free and bound DNA are indicated. The products were separated on a 4% native polyacrylamide gel and visualized by autoradiography. (B) quantification of rhRPA binding undamaged (open bars) or cisplatin-damaged (solid bars) DNA in the absence or presence of 2 mM $MgCl_2$. Quantification was performed by PhosphorImager analysis as described in Experimental Procedures, and the results presented are the average and standard deviation from three individual experiments.

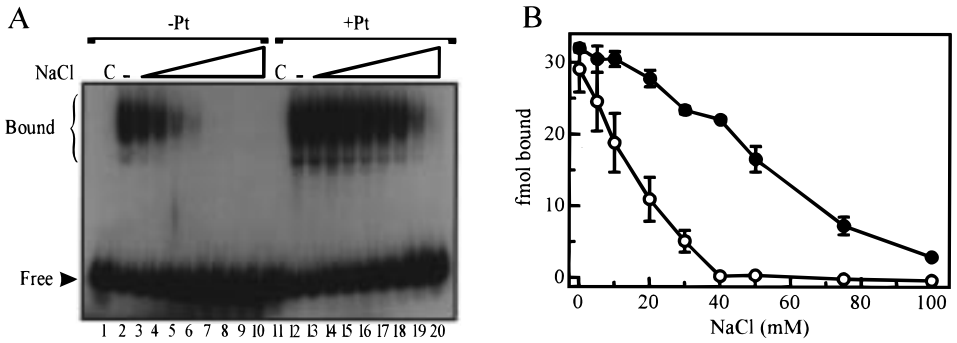

FIGURE 3: Titration of NaCl abolishes rhRPA binding to duplex undamaged DNA while minimally affecting binding to damaged DNA. (A) EMSAs were performed with 50 fmol of either undamaged (lanes 1−10) or cisplatin-damaged (lanes 11−20) duplex 44-bp DNA and 50 ng (425 fmol) of rhRPA (lanes 2−10 and 11−20) and the indicated level of NaCl: lanes 1, 2, 11, and 12, without added NaCl; lanes 3 and 13, 5 mM NaCl; lanes 4 and 14, 10 mM NaCl; lanes 5 and 15, 20 mM NaCl; lanes 6 and 16, 30 mM NaCl; lanes 7 and 17, 40 mM NaCl; lanes 8 and 18, 50 mM NaCl; lanes 9 and 19, 75 mM NaCl; lanes 10 and 20, 100 mM NaCl. (B) Quantification of rhRPA binding undamaged (○) or cisplatin-damaged (●) duplex DNA with increasing concentrations of NaCl. The results presented are the average of two individual experiments and error bars represent the range of values.

44-bp DNA, which results in a minimal binding of RPA over the range of concentrations tested. In contrast, a linear increase in RPA binding was observed with the cisplatin-treated DNA (lanes 6−10). The free and bound forms of DNA are indicated by an arrowhead and a bracket, respectively. The addition of increasing concentrations of rhRPA also resulted in the formation of multiple complexes with the damaged DNA. These multiple complexes represent different forms of rhRPA−DNA and could be a heterogeneous population of rhRPA bound to double-stranded DNA, two molecules of RPA bound per molecule of DNA (*26*), a difference in migration between the 53 kDa proteolytic fragment and the 70 kDa subunit of rhRPA bound to DNA, or even the generation of and binding to single-stranded DNA (*27*). Quantification of the gel is shown in Figure 5B and demonstrates that at each concentration rhRPA has a 10−20-fold enhanced binding to cisplatin-damaged duplex DNA.

Site-Specific Cisplatin-Damage Recognition by rhRPA. The increased binding of rhRPA to the globally damaged 44-bp DNA could be the result of a specific cisplatin−DNA adduct or a disrupted DNA structure caused by multiple cisplatin−DNA adducts. To determine if the enhanced binding of RPA to cisplatin-damaged DNA is the result of a specific cisplatin−DNA adduct, a substrate was designed containing a single 1,2-d(GpG) cisplatin−DNA adduct as described in Experimental Procedures. Results showing rhRPA binding to a fully duplex 25-bp DNA in the presence or absence of a single site-specific 1,2-d(GpG) cisplatin−DNA adduct is presented in Figure 6A. Lanes 1−5 represent the control undamaged 25-bp duplex DNA and reveals a low level of rhRPA binding compared to the cisplatin-damaged 25-bp duplex DNA in lanes 6−10. Quantification of the gel shows a 4−6-fold increase in rhRPA binding to the cisplatin-treated DNA compared to the undamaged control DNA in the presence of 50 mM NaCl and 2 mM $MgCl_2$ (Figure 6B). The level of preferential binding to a single cisplatin−DNA adduct can be increased significantly by further reducing the nonspecific binding to the undamaged

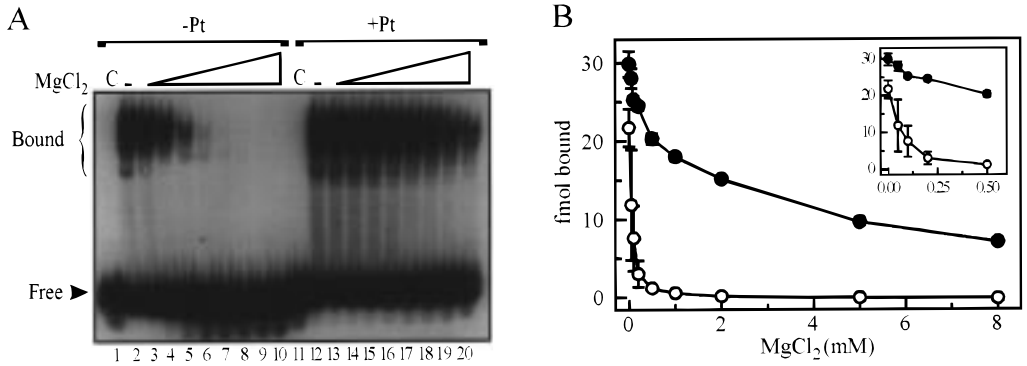

FIGURE 4: Specificity of RPA for cisplatin-damaged DNA is accentuated by $MgCl_2$. (A) EMSAs were performed with 50 fmol of either undamaged (lanes 1−10) or cisplatin-damaged (lanes 11−20) duplex 44-bp DNA and with 50 ng (425 fmol) of rhRPA (lanes 2−10 and 11−20) and the indicated amount of $MgCl_2$: lanes 1, 2, 11 and 12, without added $MgCl_2$; lanes 3 and 13, 0.05 mM $MgCl_2$; lanes 4 and 14, 0.1 mM $MgCl_2$; lanes 5 and 15, 0.2 mM $MgCl_2$; lanes 6 and 16, 0.5 mM $MgCl_2$; lanes 7 and 17, 1 mM $MgCl_2$; lanes 8 and 18, 2 mM $MgCl_2$; lanes 9 and 19, 5 mM $MgCl_2$; lanes 10 and 20, 8 mM $MgCl_2$. The positions of free and bound DNA are indicated. (B) Quantification of rhRPA binding undamaged (○) or cisplatin-damaged (●) duplex DNA with increasing concentrations of $MgCl_2$. The results presented are the average of two individual experiments and error bars represent the range of values.

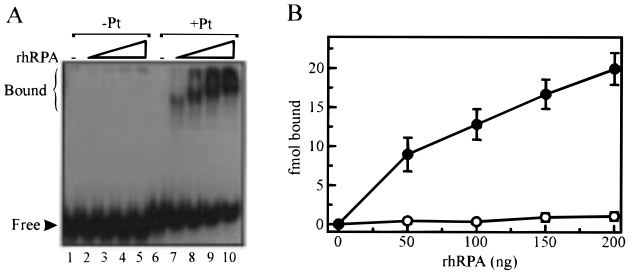

FIGURE 5: Concentration dependence of rhRPA binding a cisplatin-damaged duplex 44-bp DNA. (A) Increasing concentrations of rhRPA were incubated with 50 fmol of undamaged (lanes 1−5) or cisplatin-damaged (lanes 6−10) DNA in the presence of 50 mM NaCl and 2 mM $MgCl_2$, and the products were separated and visualized as described in Experimental Procedures. Lanes 1 and 6, without added rhRPA; lanes 2 and 7, 50 ng (425 fmol); lanes 3 and 8, 100 ng (850 fmol); lanes 4 and 9, 150 ng (1.27 pmol); lanes 5 and 10, 200 ng (1.7 pmol). (B) Quantification of increasing concentrations of rhRPA binding undamaged (○) or cisplatin-damaged (●) duplex 44-bp DNA in the presence of 2 mM $MgCl_2$ and 50 mM NaCl. Quantification was performed as described under Experimental Procedures; the results presented are the average of two individual experiments, and error bars represent the range of values.

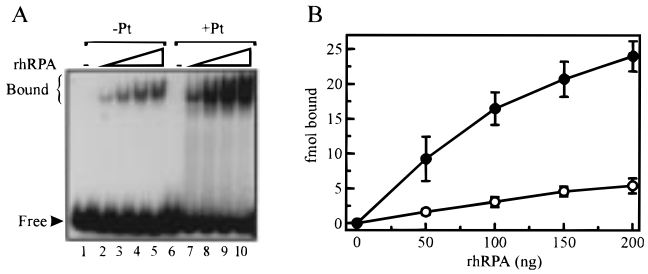

FIGURE 6: Preferential binding of RPA to duplex 25-bp DNA containing a single cisplatin adduct. The duplex 25-bp DNA containing a 1,2-d(GpG) cisplatin adduct was constructed as described under Experimental Procedures. (A) EMSAs were performed with the indicated amount of rhRPA and 50 fmol of either undamaged (lanes 1−5) or cisplatin-damaged (lanes 6−10) DNA in the presence of 2 mM $MgCl_2$ and 50 mM NaCl. Lanes 1 and 6, without added rhRPA; lanes 2 and 7, 50 ng (425 fmol); lanes 3 and 8, 100 ng (850 fmol); lanes 4 and 9, 150 ng (1.27 pmol); lanes 5 and 10, 200 ng (1.7 pmol). The products were separated on a 6% native polyacrylamide gel. (B) Quantification of increasing rhRPA concentrations binding undamaged (○) or cisplatin-damaged (●) duplex 25-bp DNA in the presence of 2 mM $MgCl_2$ and 50 mM NaCl. The results presented are the average and standard deviation from three individual experiments.

substrate. This can be achieved by increasing the length of the substrate, thus creating enhanced duplex stability, which decreases the amount of rhRPA bound to the undamaged duplex DNA while having no effect on binding to the same DNA with a single cisplatin−DNA adduct (data not shown). These data demonstrate that a single 1,2-d(GpG) cisplatin adduct is sufficient to result in increased rhRPA binding. The increased binding may be the result of the distortion in the DNA duplex structure caused by the cisplatin 1,2-d(GpG) adduct, as has been previously reported (*28*, *29*), and supports a role for RPA in the initial recognition of cisplatin-damaged DNA and possibly in mediating the subsequent nucleotide excision repair reactions.

Competition Binding of rhRPA and HMG-1 to Cisplatin-Damaged Duplex DNA. HMG-1 has been shown to bind DNA damaged with cisplatin, and binding can block the incision of the damaged DNA by NER proteins and helicase-catalyzed displacement of damaged DNA (*30*, *20*). Inhibition of the NER incision events could be the result of blocking the initial recognition of the damaged DNA or directly inhibiting the two nucleases, XPG and XPF-ERCC1. To address whether HMG-1 could block the ability of rhRPA to bind cisplatin-damaged DNA, competitive EMSA analyses were performed with the highly purified, mung bean nuclease-treated cisplatin-damaged duplex 44-bp DNA (Figures 7−9). Prebinding a constant amount of rhRPA to the cisplatin-damaged duplex DNA and subsequently adding increasing amounts of HMG-1 resulted in no change in the amount of rhRPA−cisplatin-damaged DNA complex (bracket in Figure 7A). An increase in the amount of HMG-1−cisplatin-damaged DNA complex (indicated by the arrow) was observed and represents binding to the free cisplatin-damaged DNA. Similarly, prebinding increasing amounts of rhRPA to damaged DNA resulted in a linear increase in the amount of the rhRPA-damaged DNA complex. When constant levels of HMG-1 were added to these reactions, a decrease in the amount of HMG-1 bound was observed and is the result of a lower concentration of free damaged DNA

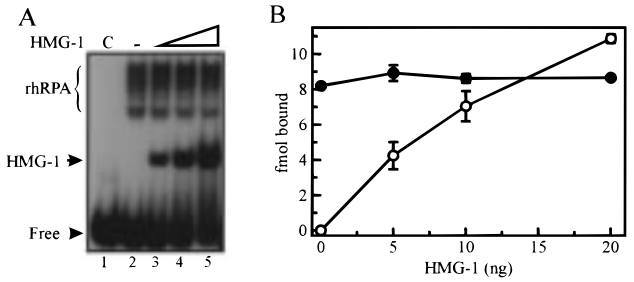

FIGURE 7: HMG-1 has no effect on rhRPA dissociation from a cisplatin-damaged duplex 44-bp DNA. (A) EMSAs were performed with 50 fmol of DNA in reactions containing 50 mM NaCl, 2 mM $MgCl_2$, and prebinding 50 ng (425 fmol) of rhRPA for 30 min. Increasing concentrations of HMG-1 were then added and incubation continued for 30 min. Products were separated as described in Experimental Procedures. Lane 1, without added HMG-1 or rhRPA; lane 2, without HMG-1; lane 3, 5 ng (180 fmol) of HMG-1; lane 4, 10 ng (360 fmol) of HMG-1; lane 5, 20 ng (720 fmol) of HMG-1. The products were separated on a 6% native polyacrylamide gel. (B) Quantification of HMG-1 (○) and rhRPA (●) binding fully duplex cisplatin-damaged 44-bp DNA. The results presented are the average of two individual experiments, and error bars represent the range of values.

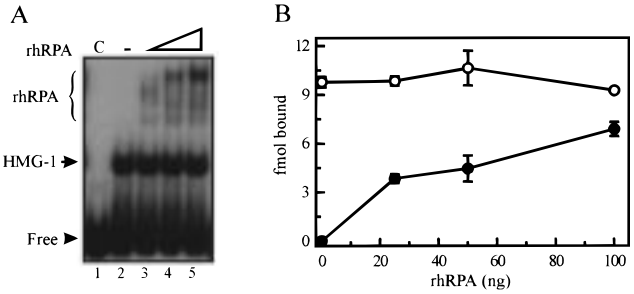

FIGURE 8: rhRPA has no effect on HMG-1 dissociation from a cisplatin-damaged duplex 44-bp DNA. (A) EMSAs were performed as described under Experimental Procedures with 50 mM NaCl, 2 mM $MgCl_2$, and prebinding 10 ng (360 fmol) of HMG-1 for 30 min. Varying concentrations of rhRPA were then added and incubation was continued for 30 min. Lane 1, without added HMG-1 or rhRPA; lane 2, without rhRPA; lane 3, 25 ng (212 fmol) of rhRPA; lane 4, 50 ng (425 fmol) of rhRPA; lane 5, 100 ng (850 fmol) of rhRPA. The products were separated on a 6% native polyacrylamide gel. (B) Quantification of the competition between HMG-1 (○) and rhRPA (●) binding duplex cisplatin-damaged 44-bp DNA. The results presented are the average of two individual experiments, and error bars represent the range of values.

in the reactions (data not shown). In reactions containing more rhRPA, such that 80% of the input DNA is bound, the addition of HMG-1 still showed no effect on the rhRPA-damaged DNA complex (data not shown). These results suggest that HMG-1 cannot displace a bound rhRPA from cisplatin-damaged DNA.

To determine if rhRPA could displace HMG-1 from cisplatin-damaged DNA, the converse experiment was performed. Constant levels of HMG-1 were prebound to cisplatin-damaged DNA and increasing concentrations of rhRPA were subsequently added. Again, there was no change in the amount of HMG-1−damaged DNA complex (arrow), and the rhRPA bound the free damaged DNA (Figure 8). The addition of rhRPA to levels that bind 100% of the remaining free DNA resulted in essentially no decrease in the amount of HMG-1−DNA complex (data not shown). These results demonstrate that RPA is unable to displace an

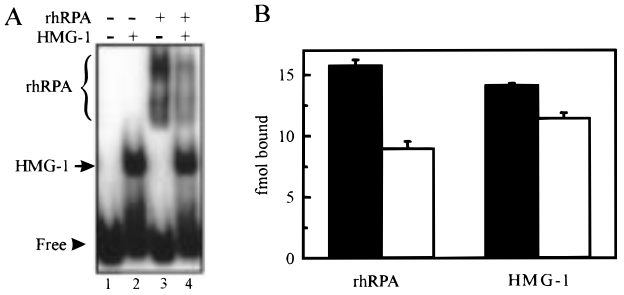

FIGURE 9: Association kinetics of HMG-1 and rhRPA with a duplex cisplatin-damaged 44-bp DNA. (A) EMSAs were performed with 50 fmol of DNA and binding was initiated by the addition of either rhRPA, HMG-1, or a mixture of these proteins. Incubation was continued for 30 min and products were separated as described in Experimental Procedures. Lane 1, without added protein; lane 2, 10 ng (360 fmol) of HMG-1; lane 3, 100 ng (850 fmol) of rhRPA; lane 4, 10 ng (360 fmol) of HMG-1 and 100 ng (850 fmol) of rhRPA. (B) Quantification of rhRPA and HMG-1 binding cisplatin-damaged DNA either alone (solid bars) or in combination (open bars). The results presented are the average of two individual experiments, and error bars represent the range of values.

HMG-1 protein from a cisplatin-damaged duplex DNA. HMG-1 had no effect on RPA binding to undamaged duplex DNA substrates, as HMG-1 does not bind to undamaged DNA substrates under these conditions (*17*).

Given the above results that neither HMG-1 nor RPA could effectively displace the other, the question arose as to what determines which protein will bind to the cisplatin-damaged DNA substrate. Therefore, we performed an experiment where the two proteins were preincubated together in the absence of DNA and then damaged DNA was added (Figure 9). The results show that the amount of rhRPA bound to the damaged DNA decreased when compared to reactions performed without preincubation with HMG-1 (lanes 3 and 4). Interestingly, the amount of HMG-1 bound to damaged DNA was minimally affected by having been preincubated with rhRPA (lanes 2 and 4). In reactions performed at near-saturating concentrations of HMG-1, rhRPA binding is nearly abolished when the two proteins are incubated simultaneously with the damaged DNA, while HMG-1 binding is minimally affected under conditions with excess rhRPA (data not shown). These results lead to a number of interesting possibilities including a difference in the association rate of HMG-1 binding to the duplex damaged DNA being greater than that of rhRPA. In addition, a potential protein−protein interaction could account for the difference in binding ability in reactions initiated following preincubation of the two proteins together. Clearly, additional experimentation is necessary to distinguish among these possibilities.

DISCUSSION

RPA in conjunction with XPA has been implicated in the initial step in the NER pathway, the recognition of damaged DNA. These proteins have also been suggested to recruit other repair proteins to the damaged site in order to mediate the subsequent NER reactions: excision, displacement, and resynthesis. Support for RPA being involved in the initial recognition events has been obtained in vitro, where RPA displays a higher affinity for UV-damaged duplex DNA in reactions containing $MgCl_2$ (*11*). Consistent with the damage

specificity of the NER process, a protein complex that specifically bound a cisplatin-damaged duplex DNA was attributed to RPA (*12*). Support for RPA's role in mediating the interaction between repair proteins was obtained by demonstrating a direct interaction with the XPA protein in the absence of DNA (*5*, *9*, *10*). In addition, RPA has been shown to interact with other repair proteins including XPG and XPF-ERCC1 (*5*, *31*, *32*). The XPA protein also binds to a wide variety of damaged DNA substrates, independent of RPA (*7*). Interestingly, RPA and XPA displayed cooperative binding to DNA damaged with *N*-acetoxy-2-acetylaminofluorene (*5*). RPA also enhances the binding and nuclease activities of the repair proteins XPG and XPF-ERCC1 to bubble- and loop substrates (*31*).

We have employed a cisplatin-damaged DNA affinity column to isolate proteins that may play a role in the metabolism of cisplatin−DNA adducts. We have identified and purified a protein complex, DRP-3, that binds cisplatin-damaged DNA. On the basis of the subunit molecular masses that copurify with the DRP-3 DNA binding activity, the similarity between the DRP-3−DNA complex and rhRPA−DNA complex, and EMSA supershift analysis using a monoclonal antibody to the 32 kDa subunit of hRPA, we have confirmed that DRP-3 is hRPA. We show that rhRPA displays a 10−20-fold increase in the preferential binding to a duplex globally cisplatin-damaged 44-bp DNA compared to the undamaged control DNA. RPA also has about a 4−6-fold higher affinity for a duplex 25-bp DNA containing a single 1,2-d(GpG) cisplatin−DNA adduct compared to the same untreated substrate. These results are consistent with a 50-fold preferential binding of rhRPA to 6−4 photoproducts (*11*) when the relative excision efficiency of the human excinuclease for these two types of damage is compared (*33*). These results suggest that RPA may initiate the first step of the NER process, the recognition of the damaged DNA. A protein that is capable of promoting local denaturation of duplex DNA in order to regulate and target the other repair proteins to the damaged site seems a likely characteristic. The ability of RPA to bind and denature duplex DNA and its ability to interact with the other NER proteins is consistent with this hypothesis (*5*, *27*, *31*). It will be interesting to determine how the XPA protein affects the ability of RPA to bind and denature cisplatin-damaged DNA and the effect the complex has on the recruitment of the other NER proteins.

A variety of non-NER proteins that bind cisplatin-damaged DNA have been purified and characterized. These proteins may also play a role in the metabolism of cisplatin−DNA adducts and could have an effect on cellular sensitivity to cisplatin. A set of these proteins includes the HMG domain proteins. IXR1 is a yeast HMG box protein that binds cisplatin-damaged DNA and has been shown to play a role in cisplatin sensitivity (*34*−*36*). HMG-1 has been shown in vitro to block the excision of cisplatin-damaged DNA, and the third step in the NER process, the helicase-catalyzed displacement of the damaged strand (*30*, *20*). The HMG-1 inhibition observed in the incision experiments could be the result of HMG-1 blocking the first step of the NER process, the recognition of the DNA damage. Disruption of the initial recognition would likely result in an inhibition of the subsequent steps, the incision of the damaged DNA by the XPG and the XPF-ERCC1 proteins. We show that HMG-1 can compete with RPA binding cisplatin-damaged DNA, suggesting that HMG-1 can block the initial recognition of cisplatin-damaged DNA.

A difference in the binding of rhRPA and HMG-1 was observed in the competition studies (Figure 9), which could be explained by a difference in association rate. The relatively slow binding of rhRPA could be the result of a two-step process, the first step being the recognition of a structural change in the DNA, followed by denaturation of the thermally unstable damaged region of DNA (*28*). Following the initial melting of this region, RPA then binds with a high affinity to the single-stranded regions. This mechanism is supported by studies demonstrating that RPA can separate two undamaged complementary DNA strands in vitro (*27*). The binding of HMG-1 to cisplatin-damaged DNA, on the other hand, is a single-step process. The structural distortion induced by a single cisplatin−DNA adduct (*29*) is similar to that induced by an HMG box binding DNA (*37*) and therefore already in a conformation capable of binding HMG-1. A detailed kinetic study of the interactions of these proteins with cisplatin-damaged DNA should provide insight into the mechanisms of protein−DNA binding. In addition, we cannot rule out the possibility that a direct interaction between RPA and HMG-1 may alter the binding of RPA to damaged DNA substrates without affecting the ability of HMG-1 to bind to damaged DNA. The effect of HMG-1 on the RPA−DNA binding activity and the interaction between RPA and XPA remains to be determined. The role these proteins play in the metabolism of cisplatin−DNA adducts is currently under investigation.

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