Replication Protein A (RPA) Binding to Duplex Cisplatin-damaged DNA Is Mediated through the Generation of Single-stranded DNA*

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Replication protein A (RPA) is a heterotrimeric protein composed of 70-, 34-, and 14-kDa subunits that has been shown to be required for DNA replication, repair, and homologous recombination. We have previously shown preferential binding of recombinant human RPA (rhRPA) to duplex cisplatin-damaged DNA compared with the control undamaged DNA (Patrick, S. M., and Turchi, J. J. (1998) Biochemistry 37, 8808–8815). Here we assess the binding of rhRPA to DNA containing site-specific cisplatin-DNA adducts. rhRPA is shown to bind 1.5–2-fold better to a duplex 30-base pair substrate containing a single 1,3d(GpXpG) compared with a 1,2d(GpG) cisplatin-DNA intrastrand adduct, consistent with the difference in thermal stability of DNA containing each adduct. Consistent with these data, a 21-base pair DNA substrate containing a centrally located single interstrand cisplatin cross-link resulted in less binding than to the undamaged control DNA. A series of experiments measuring rhRPA binding and concurrent DNA denaturation revealed that rhRPA binds duplex cisplatin-damaged DNA via the generation of single-stranded DNA. Single-strand DNA binding experiments show that rhRPA binds 3–4-fold better to an undamaged 24-base DNA compared with the same substrate containing a single 1,2d(GpG) cisplatin-DNA adduct. These data are consistent with a low affinity interaction of rhRPA with duplex-damaged DNA followed by the generation of single-stranded DNA and then high affinity binding to the undamaged DNA strand.

Cisplatin, cis-diaminedichloroplatinum(II), is a chemotherapeutic drug used to treat a variety of cancers. A major clinical limitation of cisplatin treatment is the development of cellular resistance (1, 2). The cytotoxic action is believed to involve the formation of covalent DNA-adducts which block the enzymes involved in replication and transcription (reviewed in Ref. 3). The drug forms 1,2d(GpG), 1,2d(ApG), and 1,3d(G-pXpG) intrastrand adducts as well as interstrand DNA cross-links (4, 5). Each adduct bends and distorts the DNA structure in a unique manner (5–9) and has been suggested to be recognized and repaired at different rates (10). These unique DNA structural distortions induced by the cisplatin adducts are believed to be responsible for the differential repair and may individually influence protein recognition, which could ultimately lead to differential metabolism of damaged DNA. Proteins that bind to cisplatin-damaged DNA have been implicated as important components in the sensitivity of cells to cisplatin treatment (4). By understanding how each individual DNA adduct is repaired and understanding the mechanisms of protein recognition, the development of better DNA-targeted chemotherapeutic drugs is possible.

The nucleotide excision-repair (NER)† pathway is responsible for the removal of intrastrand cisplatin-DNA adducts as well as other bulky adduct damage and must display a higher affinity for damaged DNA in the vast background of undamaged DNA (reviewed in Ref. 11). The use of xeroderma pigmentosum cell lines, which are deficient in NER, has led to the isolation and characterization of the human proteins required for NER (12). The entire NER pathway has now been reconstituted using purified proteins (13, 14). The XPA and RPA proteins have been implicated in the initial recognition of damaged DNA and have been shown to interact in the absence of DNA (15, 16). Independently, both XPA and RPA have been shown to exhibit preferential binding to damaged DNA and in conjunction a synergistic effect on binding was observed (17–19). Recently, the p34 subunit of RPA has been shown to be responsible for the XPA interaction and stimulation of XPA UV-damaged DNA binding (20). Support for RPA mediating the subsequent NER reactions has been obtained in vitro where RPA interacts with the XPG and XPF-ERCC1 proteins (19, 21, 22). It has also been shown that RPA can enhance the binding and nuclease activities of XPG and XPF-ERCC1 to bubble and loop substrates (21). Other reports suggest that RPA may protect the nondamaged strand from incision by XPG and XPF-ERCC1 during NER (23, 24). The mechanism of damaged strand incision discrimination, however, has not been defined. Our data support a role for RPA regulating the incision of the damaged DNA strand by binding the single-stranded DNA opposite the DNA adduct. This would support the hypothesis that RPA protects the undamaged strand from cleavage by XPG and XPF-ERCC1.

We have previously purified a protein that binds cisplatin-damaged DNA, DRP-3 (25). Comparison of the protein subunits, the complexes formed between DNA, and EMSA supershift analysis confirmed that DRP-3 is RPA. We have also shown, by EMSA analysis, that rhRPA preferentially binds duplex cisplatin-damaged DNA compared with the undamaged control DNA dependent upon salt concentration (25). Here we show that rhRPA binding increases with moderate levels of

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† The abbreviations used are: NER, nucleotide excision repair; RPA, replication protein A; rhRPA, recombinant human replication protein A; EMSA, electrophoretic mobility shift assay; DRP, damage recognition proteins; D/N, drug to nucleotide ratio; XP, xeroderma pigmentosum; bp, base pair(s); I.C., interstrand cross-link.
cisplatin-DNA damage before excess DNA damage results in decreased rhRPA binding. Increasing cisplatin-DNA damage also results in the formation of cisplatin-DNA interstrand cross-links. We have assessed binding and denaturation to a series of substrates containing site-specific cisplatin-DNA adducts and have correlated binding with denaturation. Experiments using bubble substrates and single-stranded DNA suggest that RPA recognizes the structural distortion in the DNA and not specifically the cisplatin-DNA adduct. These data support the hypothesis that RPA binds duplex-damaged DNA, denatures the unstable region near the adduct, and then binds the single-stranded DNA opposite the adduct to regulate DNA repair. These results are discussed with respect to RPA binding and the regulation of cisplatin-DNA repair.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and purified by 15% polyacrylamide, 7 M urea preparative sequencing gel electrophoresis. Radiolabeled nucleotides were from NEN Life Science Products and unlabeled nucleotides were from Amersham Pharmacia Biotech. T4 polynucleotide kinase and [γ-32P]ATP were purchased from U. S. Biochemical Corp. Sequenase (version 2.0) was purchased from U. S. Biochemical Corp. Mung bean nuclease was from Life Technologies, Inc. Cisplatin was purchased from Sigma. All other reagents were from the standard suppliers.

**Protein Purification**—The rhRPA expression vector was kindly provided by Marc Wool and purified as described previously (26).

**DNA Substrates**—The duplex 44-bp DNA was prepared by annealing the complementary 25-mer depicted in Table I and extended using Sequenase, [α-32P]dGTP, and dNTPs (27). The substrate was subsequently purified on a 15% native gel by treatment with mung bean nuclease to ensure that no contaminating single-stranded DNA was present and purified by spin column chromatography (25). The platination reactions were separate for each individual substrate and described in the platination section below. The 30-base DNA oligonucleotides used to prepare the 1,2d(GpG) and 1,3d(GpXpG) substrates as well as the sequences for the other oligonucleotides are shown in Table I. The 30-bp DNA substrates were 5'-labeled using T4 polynucleotide kinase and [γ-32P]ATP and, following platination, annealed to the complementary oligonucleotides, which are depicted as either 1,2-C or 1,3-C in Table I. The 24-base DNA substrates were 5'-labeled using T4 polynucleotide kinase and [γ-32P]ATP (28). The 24-bp DNA with a single 1,2d(GpG) cisplatin-DNA adduct was 5'-labeled using T4 kinase and [γ-32P]ATP, platinated, and purified on a 15% sequencing gel to separate the damaged DNA from the DNA reacted with cisplatin (29). All the undamaged DNA and DNA substrates were prepared identically except for the omission of cisplatin.

**DNA Platination**—The 44-bp DNA substrates were treated with varying concentrations of cisplatin ranging from D/N ratios of 0.1:1–5:1 in 1 mM NaHPO4 (pH 7.5) and 3 mM NaCl for 16–20 h at 37 °C in the dark. Drug to nucleotide ratios refer to the molar amount of cisplatin to the molar amount of nucleotides of a given substrate. The DNA was purified from unreacted cisplatin by G-50 spin column chromatography or ethanol precipitation followed by washing with 70% ethanol (30). The 30-base DNA substrates (1,2 and 1,3) were 5'-labeled with [γ-32P]ATP and T4 polynucleotide kinase, platinated at 1:1 D/N ratios, and annealed to the complementary oligonucleotide following treatment with either HaeIII or AvrI for the 1,2d(GpG) or 1,3d(GpXpG) cisplatin-DNA adducts, respectively, and were subsequently purified on a 15% native gel. This ensures that 100% of the substrate was platinated. The undamaged control DNA substrates were not digested with restriction enzymes. The counts per minute recovered was determined by liquid scintillation counting an aliquot of the eluate, and on the basis of the specific activity, the picomoles of DNA recovered was calculated.

**Electrophoretic Mobility Shift Assay and Denaturation Experiments**—EMSA were performed as described previously (27). Reactions were performed with 50 fmol of DNA in the presence of 50 mM NaCl and in the presence or absence of 2 mM MgCl2 as indicated in the figure legends. Reaction products were separated by either 4 or 6% native gels. Gels were dried and quantified by PhosphorImager analysis as described previously (25). For the denaturation experiments, EMSA reactions were split in half and half analyzed by EMSA analysis and the other half terminated by the addition of 0.1% Orange G dye, 25 mM EDTA, 0.5% SDS, and 5 mg of proteinase K to denature the protein-DNA interaction and allow the separation of duplex and single-stranded DNA. These products were then analyzed on 15% native gels and quantified by PhosphorImager analysis.

**RESULTS**

**Analysis of rhRPA Binding a Duplex 44-bp DNA with Increasing Cisplatin-DNA Damage**—Previously, we have shown that rhRPA preferentially binds a duplex 44-bp DNA globally treated with cisplatin at a D/N ratio of 0.2:1 compared with the undamaged control DNA of identical sequence dependent on salt concentration (25). To determine the effect of increasing cisplatin-DNA damage on rhRPA binding, the same 44-bp DNA was treated with increasing amounts of cisplatin and binding assessed in an EMSA (Fig. 1A). The DNA was purified as described under “Experimental Procedures” and contained no contaminating single-stranded DNA as evident by native gel analysis (data not shown). The increase in cisplatin-DNA adducts distorts the DNA structure, resulting in a decreased mobility of the free DNA in the gel (lanes 1–6) (31). It was anticipated that increasing cisplatin-DNA damage would result in an increase in rhRPA binding, similar to the observation of RPA binding UV-damaged DNA (18). In reactions containing 50 mM NaCl and no MgCl2, however, rhRPA binding reached a peak at intermediate cisplatin-DNA damage before a decrease in rhRPA binding was observed (lanes 1–6). The presence of MgCl2 shifted the peak to the right, but still resulted in a decrease in rhRPA binding with higher levels of cisplatin-DNA damage (data not shown). Quantification of the binding results reveals about a 10-fold enhanced binding of rhRPA to the DNA damaged with a D/N ratio of 0.2:1 compared with the undamaged DNA (Fig. 1B), consistent with that reported previously (25). A decrease in RPA binding of 30% was observed from the peak of binding obtained at a D/N of 0.2 to that observed at a D/N of 5.

The observation that rhRPA binding decreases at higher cisplatin-DNA damage is in contradiction to the results that increased rhRPA binding was observed to DNA with increasing UV DNA damage (18). The enhanced binding of RPA to UV-damaged DNA was suggested to be the result of binding to the
RPA Binds Duplex-damaged DNA via Denaturation

**FIG. 1.** RPA binding to a duplex 44-bp DNA with increasing cisplatin-DNA adducts. *A*, EMSAs were performed using 50 fmol of duplex 44-bp DNA, 50 ng (425 fmol) of rhRPA, and buffer containing 50 mM NaCl (lanes 1–6). The duplex DNAs used in the reactions were purified as described under “Experimental Procedures” to ensure no contaminating single-stranded DNA was present. *Lane 1* is undamaged duplex DNA; *lanes 2–6* represent D/N of 0.1:1, 0.2:1, 0.5:1, 1:1, and 5:1, respectively. *B*, quantification of the rhRPA binding data from *A* is presented and represents the average of two individual experiments. The error bars represent the range of values.

**FIG. 2.** Interstrand cross-links are preferentially formed on the duplex 44-bp DNA treated at high cisplatin levels. The same duplex 44-bp DNA substrates used in Fig. 1 were separated by DNA sequencing gel electrophoresis and visualized by autoradiography as described under “Experimental Procedures.” The undamaged DNA is in *lane 1*, and *lane 2* is the undamaged DNA contaminant that was removed during the purification of the DNA substrate. *Lanes 3–7* represent D/N ratio of 0.1:1, 0.2:1, 0.5:1, 1:1, and 5:1, respectively. I.C. refers to the cisplatin-interstrand cross-linked DNA.

**FIG. 3.** A single interstrand cisplatin-DNA adduct inhibits the ability of rhRPA to bind duplex DNA. *A*, EMSAs were performed in buffer supplemented with 2 mM MgCl₂ and 50 mM NaCl using 50 fmol of 21-bp undamaged DNA (*lanes 1–5*) or the same 21-bp DNA containing a single interstrand cisplatin cross-link (*lanes 6–10*). *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 concentrations of rhRPA binding undamaged (○) or cisplatin-damaged (●) duplex 21-bp DNA. The results presented are the average of two individual experiments, and error bars represent the range of values.

Single-stranded nature in the DNA structure caused by the UV-induced (6-4) photoproduct (18). The interaction of cisplatin with DNA results in a variety of adducts, some of which increase single-strand generation and those that prevent single-stranded DNA formation. Sequencing gel analysis of the duplex 44-bp DNA (Fig. 2) shows an increase in the formation of cisplatin-DNA interstrand cross-links with increasing cisplatin-DNA damage (*lanes 1–7*). *Lane 2* represents contaminating DNA that was purified away through native gel purification and is most likely incompletely extended DNA that occurred during the substrate preparation. The covalent bond formed between the platinum and the N7 positions of the guanines on two complementary DNA strands, cisplatin-interstrand cross-link, inhibits the denaturation of the two strands and results in a complex with a molecular weight about twice that expected (indicated by the brace, I.C.). The formation of DNA interstrand cross-links could be responsible for the decreased ability of rhRPA to bind/denature the duplex 44-bp DNA. At the highest level of cisplatin-DNA damage, greater than 80% of the DNA has at least one interstrand cross-link. The sequence of the duplex 44-bp DNA reveals that the interstrand cross-links can form at both ends of the DNA at three specific dGdC sites. All three sites are within 7 bases of either terminus. This would still allow rhRPA to bind in the central region of the DNA and may be the reason why rhRPA binding is not completely inhibited at the highest level of cisplatin-DNA damage.

**rhRPA Binding Is Inhibited by Cisplatin-Interstrand Cross-links**—Considering the results correlating the percent interstrand cross-links with a decrease in rhRPA binding, we assessed binding of rhRPA to a DNA substrate containing a single site-specific, centrally located cisplatin-interstrand DNA cross-link (Fig. 3A). The 21-bp DNA was constructed as described previously (28) and purified to remove any contaminating single-stranded DNA (see “Experimental Procedures”). Approximately 95% of the substrates purified via this method contained an interstrand cross-link (data not shown). EMSA analysis revealed that increasing rhRPA concentrations resulted in a low level of rhRPA binding to the undamaged duplex control DNA substrate (*lanes 1–5*), and interestingly, even lower levels of rhRPA binding to the DNA substrate containing a single cisplatin-DNA interstrand cross-link (*lanes 5–10*). Quantification of the EMSA is shown in Fig. 3B. The high level of rhRPA binding the undamaged control DNA is likely the result of decreased stability of the 21-bp DNA. Increasing the duplex DNA length increases the DNA stability and decreases the nonspecific rhRPA binding as can be seen when comparing the undamaged 21-bp DNA, a 25-bp undamaged DNA, and the undamaged 30-bp DNA (data not shown). The inability of rhRPA to bind a substrate containing a cisplatin-interstrand cross-link is consistent with rhRPA binding occurring via denaturation of the duplex DNA. We do observe a low level of rhRPA binding to the interstrand cross-link DNA, which could be the result of the 5% contaminating duplex undamaged DNA or an extremely low affinity for DNA containing a cisplatin-interstrand cross-link.
rhRPA Binding Correlates with the Ability to Denature Cisplatin-damaged DNA—RPA has been shown to denature undamaged DNA substrates, including long stretches of duplex DNA dependent on the ionic strength (32, 33). RPA has also been shown to bind and denature DNA substrates containing an SV40 “pseudo-origin” of replication (34). In order to assess whether the denaturation ability of rhRPA was responsible for the preferential binding observed to duplex cisplatin-damaged DNA compared with duplex undamaged DNA, a series of experiments were performed with the duplex undamaged and 0:1:1 cisplatin-treated DNA substrates measuring both DNA binding (data not shown) and DNA denaturation (Fig. 4A). Separation of reaction products was achieved using a 15% native gel in the denaturation experiments. In addition, SDS and proteinase K were added to the stop buffer to denature the protein and disrupt the protein-DNA interaction. The 4% native polyacrylamide gel and visualized by autoradiography. Lanes 1 and 6, without added rhRPA; lanes 2 and 7, 25 ng (212 fmol); lanes 3 and 8, 50 ng (425 fmol); lanes 4 and 9, 100 ng (850 fmol); lanes 5 and 10, 200 ng (1.7 pmol). B, quantification of increasing concentrations of rhRPA denaturing (● and ○) and binding (●, ○) the undamaged (△ and □, respectively) and the cisplatin-damaged (● and ○, respectively) 44-bp DNA in the presence of 50 mM NaCl.

rhRPA Binds Preferentially to Cisplatin-DNA Intrastrand Adducts—If rhRPA binding is proportional with the thermal instability of the DNA duplex, DNA substrates with varying thermal stabilities should result in varying degrees of binding by RPA. Therefore, DNA substrates were designed containing either a single site-specific 1,2d(GpG) or 1,3d(GpXpG) cisplatin-DNA adduct. The 1,2 adduct induces a bend and unwinds the duplex DNA, but results in no localization of single-stranded DNA (36). In contrast, the 1,3 adduct results in a localized denaturation of approximately 2 bases around the adduct (37, 38). We have shown previously a 4–6-fold preference for rhRPA binding a duplex 25-bp DNA containing a single 1,2d(GpG) cisplatin-intrastrand DNA adduct compared with the same undamaged substrate (25). Here we have designed longer substrates for rhRPA binding analysis of both types of intrastrand adducts in order to increase the stability of the DNA and decrease the nonspecific binding of rhRPA to the undamaged DNA. The 30-bp substrates used for the cisplatin-intrastrand rhRPA binding experiments were prepared as described under “Experimental Procedures” and were completely devoid of any contaminating single-stranded DNA. The binding reactions were conducted in the presence of 50 mM NaCl and 2 mM MgCl₂ to minimize damage-independent binding (Fig. 5). Increasing rhRPA concentrations resulted in minimal rhRPA binding to the undamaged 30-bp DNA (data not shown), while an increase in the amount of rhRPA bound was observed using the same substrate with a single 1,2d(GpG) cisplatin-DNA adduct (Fig. 5A, lanes 1–5). Quantification of the results reveals up to 10-fold enhanced binding of rhRPA to the 1,2d(GpG) duplex-damaged DNA compared with the undamaged control (Fig. 5B, filled and open circles, respectively). Fig. 5A also shows the EMSA analysis of increasing concentrations of rhRPA binding the same substrate with a single 1,3d(GpXpG) cisplatin-DNA adduct (lanes 6–10). Quantification reveals products destabilize the DNA structure largely independent of ionic strength.

rhRPA Binds Duplex-damaged DNA via Denaturation—Fig. 4. Denaturation of duplex 44-bp DNA by rhRPA correlates with binding activity. A, denaturation experiments were performed under identical conditions as the EMSAs using the duplex 44-bp DNA, but the reaction stop buffer contained SDS and proteinase K to disrupt the protein-DNA interaction, and the products were run on a 15% native gel to separate duplex from single-stranded DNA. Lanes 1 and 7 are the heat-denatured controls, △, for the undamaged and 0.1:1 cisplatin-damaged 44-bp DNA, respectively. Lanes 2 and 8, depicted by C are the no enzyme controls for the undamaged and cisplatin-damaged DNA. Lanes 3 and 9, 25 ng of rhRPA (212 fmol); lanes 4 and 10, 50 ng of rhRPA (425 fmol); lanes 5 and 11, 100 ng of rhRPA (850 fmol); lanes 6 and 12, 200 ng of rhRPA (1.7 pmol). B, quantification of increasing concentrations of rhRPA denaturing (● and ○) and binding (●, ○) the undamaged (△ and □, respectively) and the cisplatin-damaged (● and ○, respectively) 44-bp DNA in the presence of 50 mM NaCl.

rhRPA Binds Duplex-damaged DNA via Denaturation:—Fig. 5. rhRPA binding correlates with the instability of the cisplatin-damaged duplex DNA. A, EMSAs were performed using the following indicated amounts of rhRPA and 50 fmol of either the 30-bp 1,2 adduct (lanes 1–5) or 1,3 adduct (lanes 6–10) in the presence of 2 mM MgCl₂ and 50 mM NaCl. The products were separated on a 4% native polyacrylamide gel and visualized by autoradiography. Lanes 1 and 6, without added rhRPA; lanes 2 and 7, 25 ng (212 fmol); lanes 3 and 8, 50 ng (425 fmol); lanes 4 and 9, 100 ng (850 fmol); lanes 5 and 10, 200 ng (1.7 pmol). B, quantification of increasing rhRPA concentrations binding undamaged (○, □), 1,2d(GpG) (●), or 1,3d(GpXpG) (●) cisplatin-damaged DNA. The results presented are the average of two individual experiments, and error bars represent the range of values.
Directly to the Cisplatin-DNA Adduct—a role for RPA in the initial steps of the NER process (19). DNA intrastrand adducts (10). These data are consistent with have been shown previously for these two types of cisplatin—ultimately be responsible for the differential repair rates that differential binding of rhRPA to different DNA adducts could individual intrastrand adducts influence rhRPA binding. The suggest that the unique structural distortions induced by the denature the DNA substrate (data not shown). These data of rhRPA to the 30-bp DNAs correlates with the ability to nating single-stranded DNA (data not shown). EMSAs were performed with the indicated amounts of rhRPA in the presence of 2 mM MgCl, and 50 mM NaCl. Lanes 1 and 6, without added rhRPA; lanes 2 and 7, 25 ng (212 fmol); lanes 3 and 8, 50 ng (425 fmol); lanes 4 and 9, 100 ng (850 fmol); lanes 5 and 10, 200 ng (1.7 pmol). The products were separated on a 4% native polyacrylamide gel. B, quantification of increasing rhRPA concentrations binding a duplex 30-bp substrate with a 1,3d(GpXpG) cisplatin-DNA adduct (●), a 30-bp undamaged substrate with an 8-base bubble (■), or the same 30-bp bubble substrate with a single 1,3d(GpXpG) cisplatin-DNA adduct (○). The results presented are the average and S.D. from three individual experiments.

greater than 15-fold enhanced binding of rhRPA to the 1,3d(G-pXpG) cisplatin-DNA adduct compared with the undamaged control DNA of identical sequence in the linear ranges of the graph (Fig. 5B, filled and open squares, respectively). The quantification also reveals about a 1.5–2-fold preferential binding to the 1,3d(GpXpG) cisplatin-DNA intranstrand adduct compared with the 1,2d(GpG) cisplatin-DNA intranstrand adduct under identical binding conditions (filled squares and filled circles, respectively). As seen with the 44-bp DNA, the binding of rhRPA to the 30-bp DNAs correlates with the ability to denature the DNA substrate (data not shown). These data suggest that the unique structural distortions induced by the individual intranstrand adds influence rhRPA binding. The differential binding of rhRPA to different DNA adducts could ultimately be responsible for the differential repair rates that have been shown previously for these two types of cisplatin-DNA intranstrand adducts (10). These data are consistent with a role for RPA in the initial steps of the NER process (19).

rhRPA Binds the Structural Distortion in the DNA and Not Directly to the Cisplatin-DNA Adduct—The results from the cisplatin damage titration (Fig. 1) and the differential binding of rhRPA to substrates containing a cisplatin 1,2d(GpG) and a 1,3d(GpXpG) intrastrand DNA adduct (Fig. 5) suggest that rhRPA is binding the structural distortion and not specifically to the cisplatin-DNA adduct. To address this hypothesis, DNA substrates containing 8-base bubbles were designed to further assess the binding specificity of rhRPA on duplex DNA substrates. The 30-bp bubble substrates were prepared as described under “Experimental Procedures,” and denaturation experiments ensured the substrates were devoid of contaminating single-stranded DNA (data not shown). EMSAs were performed with 50 fmol of DNA and increasing concentrations of rhRPA under the conditions stated under “Experimental Procedures,” and the results are shown in Fig. 6A. The substrates used for the EMSA were the duplex 30-bp DNA with a single 1,3d(GpXpG) cisplatin-DNA adduct (data not shown), a 30-bp undamaged DNA with an 8-base bubble (lanes 1–5), and the same 8-base bubble substrate with a single 1,3d(GpXpG) cisplatin-DNA adduct located within the bubble region (lanes 6–10). Results from the quantification show an increase in rhRPA binding to the 30-bp DNA with a single 1,3d(GpXpG) cisplatin-DNA adduct (filled circles) consistent with that presented earlier (Fig. 5B). The binding of rhRPA to the undamaged 8-base bubble substrate (filled squares), however, is 3–4-fold better than to the 30-bp DNA with the single 1,3d(GpXpG) cisplatin-DNA adduct (filled circles). Interestingly, the level of rhRPA binding to the 8 base bubble substrate with a 1,3d(G-pXpG) cisplatin-DNA adduct (open squares) is lower than that of the undamaged 8-base bubble substrate (filled squares). This data suggests that rhRPA is binding via the structural distortion in the damaged DNA and not specifically interacting with the cisplatin-DNA adduct. It also suggests that the cisplatin-DNA adduct may inhibit the binding of rhRPA to the damaged DNA strand.

rhRPA Preferentially Binds an Undamaged Single-stranded DNA Compared with the Same DNA with a Single 1,2d(GpG) Cisplatin-DNA Adduct—To better understand the binding mechanism of rhRPA to cisplatin-damaged DNA, a single-stranded 24-mer DNA was used in an EMSA to test the hypothesis that rhRPA binding is inhibited by a cisplatin-DNA adduct on single-stranded DNA (open circles). Increasing rhRPA concentrations were added to 50 fmol of an undamaged single-stranded 24-mer DNA (lanes 1–5) or the same DNA with a single 1,2d(GpG) cisplatin-DNA adduct (lanes 6–10). Quantification of the EMSA shown in Fig. 7B reveals that rhRPA binds 3–4-fold better to the undamaged single-stranded DNA (open circles) compared with the same DNA with a single 1,2d(GpG) cisplatin-DNA adduct (filled circles). This supports the hypothesis that rhRPA is binding and recognizing the DNA distortion
in duplex DNA and not specifically the cisplatin-DNA adduct. The data also support a model where following recognition of DNA damage by sensing the thermal instability of the duplex DNA, RPA binds preferentially to the undamaged strand of the duplex damaged DNA (Fig. 8).

**DISCUSSION**

The process of NER is capable of recognizing and repairing a wide array of bulky DNA damage. The rates of repair of the different DNA-adducts also vary dramatically. The limiting step in NER has been reported to be incision of the damage, which is completely dependent on recognizing the damaged DNA in a background of a vast amount of undamaged chromosomal DNA (reviewed in Ref. 11). The efficiency of NER has been correlated with the thermodynamic instability of the DNA duplex caused by the individual DNA adducts (39). As the recognition of DNA damage limits the reactions and a correlation between repair and thermal stability of the duplex has been established, one would expect that proteins involved in the initial recognition would show a preference for binding damaged DNA with a lower thermal stability. Our data support this hypothesis, and we have demonstrated that the affinity for damaged DNA correlates with the thermal instability of the duplex DNA. RPA binding to a DNA containing a single 1,3d(G-pG) cisplatin adduct is more efficient than binding to a 1,2d(GpG) cisplatin adduct (Fig. 5). These data correlate well with the degree of DNA instability caused by each adduct, since in the platinated guanines being within hydrogen bonding distance of the complementary cytosines (38, 36). Also, our previous data demonstrated that conditions that increase the stability of the duplex DNA inhibit RPA binding, for example, ionic strength of the binding reactions (25). In addition, we have shown a single interstrand cisplatin cross-link, which prevents denaturation of the duplex DNA, effectively inhibits RPA binding (Fig. 3). These data are consistent with RPA being involved in the initial recognition of cisplatin-damaged DNA and targeting repair to the damaged site.

RPA has been reported to have an affinity for duplex DNA 3 orders of magnitude less than that for single-stranded DNA (reviewed in Ref. 40). Our data demonstrate that binding observed in EMSAs proceeds via the denaturation of the duplex DNA substrate. This is consistent with the reported unwinding activity of RPA (32, 33) and our data measuring denaturation of the duplex undamaged or cisplatin-damaged DNA substrate (Fig. 4). Our data suggest that the protein-DNA complexes observed in EMSAs are in fact RPA complexes to single-stranded DNA that was generated via denaturation of the duplex DNA and that the intermediate in the reaction representing the RPA-duplex DNA is not stable through electrophoresis. Therefore, dissociation constants calculated for duplex DNA may represent the ability to denature and not actual affinity for the RPA-duplex DNA complex. Our data suggest that following RPA-induced denaturation of the duplex-damaged DNA, RPA preferentially binds to the undamaged DNA strand. This was demonstrated in reactions measuring binding of RPA to a 24-base DNA in the presence and absence of a centrally located single cisplatin-DNA adduct. While RPA binding to the platinated DNA was reduced by more than 50% when compared with the undamaged DNA, significant binding was observed to the platinated single-stranded DNA substrate. This could be the result of RPA binding to the regions of the DNA without the adduct, as 11 bases on either side of the adduct are available for high affinity binding. RPA binding to the bubble substrates also support the hypothesis that rhRPA is recognizing the structural distortion in the DNA and not the damaged bases specifically. The decrease in binding the bubble substrate containing the 1,3 adduct is less than that observed with the single-stranded 24-base DNA. This is likely the result of high molar ratio of RPA to duplex DNA necessary to denature the DNA. Once the duplex substrate is denatured, rhRPA can then bind to the single-stranded undamaged strand, which is not labeled and is never detected, as well as to the labeled single-stranded damaged DNA strand. The excess rhRPA could then bind the single-stranded DNA flanking the damaged site, which is 14 bases on either side, resulting in the observed RPA-DNA complex. Consistent with this interpretation, we have observed consistently greater binding activity to the duplex cisplatin-damaged DNA substrate when the complementary, undamaged DNA strand is labeled (data not shown). These data represent the first direct evidence that RPA regulates DNA repair by binding to duplex-damaged DNA, locally denaturing the duplex DNA as a result of the DNA damage, and then binding to the undamaged DNA strand.

Our model positioning RPA on the undamaged DNA is consistent with a recent report demonstrating that RPA binds single-stranded DNA with a defined polarity and inhibits cleavage by the XPG and the XPF-ERCC1 endonucleases on the strand that RPA is bound (24). The crystal structure of RPA bound to an oligonucleotide also suggests some level of RPA binding polarity (41). Positioning of RPA on the undamaged DNA strand and the defined polarity of binding also positions the other repair factors in the proper orientation for their known enzymatic activities. The N terminus of the 70-kDa subunit of RPA interacts with XPG (19), and the 34-kDa subunit of RPA interacts with the XPA protein (20). The XPF protein has also been shown to interact with RPA (22). These interactions would place XPG 3' of the cisplatin adduct where it has been shown to incise and the XPF-ERCC1 nuclease 5' of the adduct where it has been shown to incise (reviewed in Ref. 11). In addition, binding the undamaged strand by RPA would protect the strand from nuclease incision as has been demonstrated using undamaged synthetic DNA substrates. It will be interesting to determine the possible strand specificity of XPA protein binding when complexed with RPA. In addition, how the TFIIH and XPC-HHR23B components of the NER machinery fit in this model remains to be determined.

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