Xeroderma Pigmentosum Complementation Group A Protein (XPA) Modulates RPA-DNA Interactions via Enhanced Complex Stability and Inhibition of Strand Separation Activity*

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Replication protein A (RPA) participates in many cellular functions including DNA replication and nucleotide excision repair. A direct interaction between RPA and the xeroderma pigmentosum group A protein (XPA) facilitates the assembly of a preincision complex during the processing of DNA damage by the nucleotide excision repair pathway. We demonstrate here the formation of a ternary RPA, XPA, and duplex cisplatin-damaged DNA complex as evident by electrophoretic supershift analysis. The RPA-XPA complex displays modest specificity for damaged versus undamaged duplex DNA, and the RPA-XPA complex displays a greater affinity for binding duplex cisplatin-damaged DNA when compared with the RPA or XPA proteins alone, consistent with previous results. Using DNA denaturation assays, we demonstrate that the role of XPA is in the stabilization of the duplex DNA structure via inhibition of the strand separation activity of RPA. Rapid kinetic analysis indicates that the bimolecular $k_{on}$ of the RPA-XPA complex is 2.5-fold faster than RPA alone for binding a duplex cisplatin-damaged DNA. The dissociation rate, $k_{off}$, of the RPA-XPA complex is slower than that of the RPA protein alone, suggesting that the XPA protein stabilizes the initial binding of RPA to duplex DNA as well as maintaining the integrity of the duplex DNA. Interestingly, XPA has no effect on the $k_{on}$ of RPA for a single-stranded 40-mer DNA.

Nucleotide excision repair (NER) is the major pathway responsible for the removal of a wide array of bulky DNA adducts from the genome (1, 2). A defect in this pathway can result in genomic instability and also results in a predisposition to skin cancer, as is evident by the xeroderma pigmentosum (XP) disorder (3). Although the protein components have been identified and the NER reaction has been reconstituted in vitro (4, 5), the biochemical process through which the global genomic repair pathway is initiated and recognizes damaged DNA is still poorly understood. The heterotrimeric replication protein A (RPA) is required for NER and has been suggested to play a role in the damage recognition process (6–8). The XPA protein is also required for NER and is involved in the DNA damage recognition process (4, 5, 9). Both RPA and XPA preferentially bind damaged DNA, and because RPA and XPA directly interact in the absence of DNA, the RPA-XPA complex has been implicated as a key component in the earliest stage of damage recognition (9–14). There is also evidence that the XPC-hHR23B protein complex may initiate recognition of DNA damage for the global genomic repair pathway of NER (15). Recent evidence also implicates the DDB heterodimer in damage recognition because the complex binds damaged DNA with high affinity (16) and can dramatically increase the repair rate of certain DNA adducts, including cyclobutane pyrimidine dimers in conjunction with XPA and RPA (17). In addition, the p48 subunit of DDB has been demonstrated to localize to the sites of UV-induced DNA damage independent of XPA and XPC (18).

The exact role that these protein components play in recognizing damaged DNA in the vast background of undamaged DNA to initiate NER remains to be determined. The affinity of these individual protein components for damaged DNA cannot account for the specificity and selectivity required for distinguishing the damaged DNA from the background of undamaged DNA to initiate NER (1, 2). In light of recent results demonstrating that in vitro NER catalyzed incision of undamaged DNA can be detected, the degree of NER specificity for damaged DNA may be less than originally thought (19). The protein components may work in a synergistic manner to account for the damage-specific preference required, and/or the transcription machinery may alleviate some of the selectivity required by stalling at DNA lesions and recruiting the NER factors in a transcription-coupled repair mechanism. The question still remains as to what factor(s) initiates and acts as a nucleation point for NER in the absence of a stalled transcription complex. A possibility is that the NER initiator(s) could be a preformed complex of damage recognition proteins that synergistically function to selectively bind to a damaged-DNA site.

This initial complex of damage recognition proteins could then recruit the other repair factors to initiate NER. Both in vitro (20) and recent in vivo evidence (21) suggests that recognition of DNA damage proceeds via a stepwise assembly of NER.

It has been unclear whether the RPA protein and the XPA protein remain in a complex when bound to DNA and to what extent the protein-protein interaction has on the ability to recognize damaged DNA. This is evidenced by the difficulty in observing this complex or obtaining a distinct footprint on damaged DNA (14). In an attempt to better understand DNA damage recognition and the synergistic effects between protein components, we have assessed the effect of the RPA-XPA in-

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The abbreviations used are: NER, nucleotide excision repair; RPA, human replication protein A; XPA, xeroderma pigmentosum group A protein; $k_{on}$, association rate; $k_{off}$, dissociation rate; EMSA, electrophoretic mobility shift assay; ssDNA, single-stranded DNA; Ab, antibody; mAb, monoclonal antibody; DDB, damaged-DNA-binding protein; HMG, high mobility group.
Interaction on cisplatin-damaged DNA binding. We show the formation of an RPA-XPA complex bound to duplex cisplatin-damaged DNA as evident by supershift analysis by antibodies to both proteins. The RPA-XPA complex binds synergistically to duplex cisplatin-damaged DNA compared with RPA or XPA alone, and there is a specificity of the complex for duplex cisplatin-damaged DNA versus duplex undamaged DNA. The XPA protein stabilizes the duplex DNA structure and inhibits the strand separation induced by RPA. Stopped-flow kinetic analyses reveal a synergistic effect on the rate constants for binding duplex cisplatin-damaged DNA and support the hypothesis that the RPA and XPA proteins may be in complex prior to binding a damaged DNA site. These results are discussed with respect to DNA damage recognition and the regulation of nucleotide excision repair.

**EXPERIMENTAL PROCEDURES**

**Materials**—All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Cisplatin was purchased from Sigma, mung bean nuclease and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA), and sequencing (version 2.0) was purchased from U.S. Biochemical Corp. (Cleveland, OH). Radiolabeled nucleotides were from PerkinElmer Life Sciences, and unlabeled nucleotides were from Amersham Biosciences. The anti-p34 RPA monoclonal antibody (RPA/p34 Ab-1) was purchased from NeoMarkers (Fremont, CA), and the anti-Xpress, which recognizes the amino acid linker sequence between the His6 tag and the N terminus of the XPA protein, was purchased from Invitrogen. All other reagents, enzymes, and chemicals were from standard suppliers. The 40- and 41-mer DNA sequences used to prepare the duplex DNA substrates are 5′-TCATTACTCTAacctCGCCGATCGCCTTCTTATCCC-3′ and 5′-GGGGAAATAGAGAGCGATGGCCGACAGTAGTAATGGA-3′, respectively.

**Protein Purification**—Recombinant RPA was purified as described using the expression vector kindly provided by Marc Wold (22). The His6-XPA fusion protein was purified as previously described (23).

**Radiolabelled DNA Synthesis**—The DNA substrates used for electrophoretic mobility shift assay (EMSA) analysis were treated with cisplatin, labeled with 32P, and purified as previously described (11). Following gel purification, the DNA substrates were treated with mung bean nuclease to reduce the single-stranded DNA contamination to less than 1% (data not shown).

**Electrophoretic Mobility Shift and Denaturation Assays**—EMSAs were performed using 50 fmol of either undamaged or cisplatin-treated duplex 41-bp DNA in a reaction buffer containing 20 mM HEPES (pH 7.8), 2 mM dithiothreitol, 0.001% Nonidet P-40, and 50 mM NaCl (11, 24). The presence or absence of 2 mM MgCl2 and the indicated amount of each protein is shown in the figure legends. The proteins, a 4:1 molar ratio of XPA to RPA, were preincubated on ice for 15 min and then added to the DNA and incubated for an additional 15 min at room temperature. The specific antibodies were added, where indicated after the RPA or RPA-XPA proteins were incubated with the DNA and incubated for 15 additional minutes at room temperature. Gel loading buffer was added, and the products were separated on 4% Native polyacrylamide gels as previously described (11, 24). For the EMSA and denaturation assays, the reactions were split in half and analyzed separately (24). The products were then analyzed on 15% native gels to allow the separation of duplex and single-stranded DNA and quantified by phosphorimaging analysis.

**Stopped-flow Kinetic Experiments and Data Analysis**—Stopped-flow kinetic traces were obtained using a SX.18MV stopped-flow reaction analyzer (Applied Photophysics) as previously described (22). Equal volumes of protein and DNA in reaction buffer supplemented with 2 mM MgCl2 from separate syringes were rapidly mixed at 24 °C. Fluorescence was measured following excitation at 290 nm (0.5-mm slit width) using a 350-nm longpass cut-on filter (LG-350 from Corion, Franklin, MA). Constant protein (6.25 nM RPA final reaction concentration alone or preincubated with 25 nM XPA) and varying DNA concentrations (62.5–125 nM final concentrations when mixed) starting at a 10-fold molar excess of DNA to RPA were used to achieve pseudo-first-order kinetics. The RPA and XPA proteins were preincubated on ice for 15 min prior to the stopped-flow reactions. The kinetic data were fit using Pro-K software (Applied Photophysics) to calculate the observed, $k_{obs}$. The results obtained for RPA and the RPA-XPA complex binding duplex DNA were obtained modeling a double exponential decay. The

**RESULTS**

**RPA-XPA Complex Formation on Duplex Cisplatin-damaged DNA**—It has previously been shown that RPA and XPA interact in the absence of DNA and that both proteins are required for NER. However, there has been considerable difficulty in observing a trimeric RPA-XPA-DNA complex because of the small change in the mobility of RPA-DNA complex compared with RPA-XPA-DNA, and no DNA footprint has been observed for the RPA-XPA complex bound to DNA. To determine whether the complex of RPA-XPA binds to duplex cisplatin-damaged DNA, we employed an EMSA using specific antibodies to RPA and XPA (Fig. 1). The RPA protein demonstrates weak binding to the duplex cisplatin-damaged 41-bp DNA under the experimental conditions employed (lane 2). The presence of the anti-Xpress antibody (XPA Ab) supershifts the bound XPA protein and enhances the binding of the XPA protein, possibly by stabilizing the protein (lane 3). The monoclonal Ab to the p34 subunit of RPA displays no cross-reactivity with the XPA protein (lane 4). The RPA protein-DNA complex is supershifted with a mAb to the p34 subunit, and there is no cross-reactivity of the anti-Xpress antibody for the RPA protein (lanes 6 and 7, respectively). The RPA-XPA complex resulted in a shifted complex that migrated slightly slower than the RPA protein alone (lane 8 versus lane 7), although this difference was variable (data not shown). Supershift analysis using either the anti-Xpress or anti-p34 antibody of RPA shifted the band and confirmed that both XPA and RPA were bound to the duplex cisplatin-damaged DNA (lanes 9 and 10, respectively). These data indicate that a preformed RPA-XPA complex can bind duplex cisplatin-damaged DNA and that both proteins are present in the bound DNA complex. Previous studies suggested

**FIG. 1.** EMSA analysis of RPA, XPA, and the RPA-XPA complex binding a duplex cisplatin-damaged DNA. EMSAs were performed using the following indicated amounts of RPA, XPA, or the RPA-XPA complex and 50 fmol of duplex 41-bp DNA containing a single, centrally located 1,24(GpG) cisplatin-DNA adduct. The products were separated on a 4% native polyacrylamide gel and visualized by autoradiography. Lane 1, no protein; lane 2, 300 ng of XPA; lane 3, 300 ng of XPA plus anti-Xpress Ab; lane 4, 300 ng of XPA plus anti-RPA p34 mAb; lane 5, 200 ng of RPA; lane 6, 200 ng of RPA plus anti-RPA p34 mAb; lane 7, 200 ng of RPA plus anti-Xpress Ab; lane 8, 300 ng XPA and 200 ng of RPA; lane 9, 200 ng of XPA and 200 ng of RPA plus anti-Xpress Ab; lane 10, 200 ng of XPA and 200 ng of RPA plus anti-RPA p34.
the existence of an RPA-XPA-DNA complex; however, the inability to footprint the complex and inefficient supershifting of the complex with specific antibodies raised doubt as to the stability of the complex (14). The data presented in Fig. 1 demonstrate conclusively that XPA is present in the complex of RPA bound to DNA.

Damage-specific and Synergistic Binding of RPA-XPA to Duplex Cisplatin-damaged DNA—To determine the specificity of the RPA-XPA complex for duplex cisplatin-damaged DNA, competition mobility shift assays were performed using nonspecific competitor poly(dI-dC) DNA (Fig. 2A). Increasing concentrations of poly(dI-dC) were incubated in reactions with either constant RPA or RPA-XPA complex using duplex cisplatin-damaged DNA (preincubated on ice); lanes 6–9 contain increasing amounts of dI-dC competitor DNA (4, 8, and 12 ng, respectively). Quantification of RPA binding duplex cisplatin-damaged DNA (filled circles) as well as the RPA-XPA complex binding duplex cisplatin-damaged DNA (open circles) with increasing competitor dI-dC DNA.

**FIG. 2.** Specificity of the RPA-XPA complex for duplex cisplatin-damaged DNA. A, EMSAs were performed as described in the legend to Fig. 1 using 50 fmol of duplex cisplatin-damaged 41-mer with the addition of 2 mM MgCl₂. The products were separated on a 4% native polyacrylamide gel and visualized by autoradiography. Lane 1, no added protein; lanes 2–5 contain 200 ng of RPA; lanes 3, 4, and 5 contain increasing amounts of dI-dC competitor DNA (4, 8, and 12 ng, respectively); lanes 6–9 contain 200 ng of RPA and 200 ng of XPA (preincubated on ice); lanes 7, 8, and 9 contain increasing amounts of dI-dC competitor DNA (4, 8, and 12 ng, respectively). B, quantification of RPA binding duplex cisplatin-damaged DNA (filled circles) as well as the RPA-XPA complex binding duplex cisplatin-damaged DNA (open circles) with increasing competitor dI-dC DNA.

1.5-fold better DNA binding for the RPA-XPA complex compared with RPA alone. The data, however, are presented as a percent of the maximum DNA bound for the RPA-XPA complex and for RPA with no competitor DNA. In addition, the RPA-XPA complex preferentially binds duplex cisplatin-damaged DNA compared with the undamaged duplex DNA (data not shown). The RPA-preferential DNA binding results for cisplatin-damaged versus undamaged duplex DNA are consistent with previously published data (11, 22, 24). Interestingly, the XPA protein also enhanced the binding of RPA to undamaged duplex DNA (data not shown). These results reveal damage-specific binding as well as synergic binding of the RPA-XPA complex to duplex cisplatin-damaged DNA when compared with the binding of the RPA or XPA proteins alone.

**XPA Inhibits RPA-induced DNA Denaturation**—We have previously demonstrated that the ability of RPA to bind duplex DNA correlates with the ability to denature duplex DNA (24). To assess the effect of XPA on the ability of RPA to unwind or denature DNA, we performed binding/denaturation assays (Fig. 3). The EMSA reveals an increase in DNA binding with increasing concentrations of either RPA or RPA-XPA (Fig. 3A, lanes 2–5 and 6–9, respectively). The presence of the anti-Xpress antibody demonstrated by the supershifted complex that XPA is present in the bound complex (Fig. 3A, lane 10). The denaturation analysis (Fig. 3B) reveals an increase in single-stranded DNA with increasing RPA (lanes 2–5) similar to our previous report (24). Single-stranded DNA is also generated with increasing concentrations of the RPA-XPA complex, but the level of single-stranded DNA is reduced in the presence of the XPA protein (lanes 6–9). The quantification (Fig. 3C) reveals a correlation between RPA binding/denaturation, consistent with previously published data (filled symbols). The presence of the XPA protein stabilizes the duplex DNA structure and results in about a 60–70% reduction in single-stranded DNA generation (open symbols). Considering the 41-bp DNA substrate is near the minimal size DNA to support RPA-XPA complex formation, the lack of complete inhibition is not surprising. These results as well as our previously published data support a model for the recognition of damaged DNA in which the RPA-XPA complex first binds duplex-damaged DNA followed by DNA strand melting and the positioning of RPA to the undamaged single-stranded DNA opposite the lesion (25). The XPA protein, via contacts with both DNA strands, inhibits further DNA denaturation and positions the protein complex near the DNA lesion (25).

**Stopped-flow Kinetic Analysis of the RPA-XPA Complex Binding Duplex Cisplatin-damaged DNA**—To better understand the DNA binding kinetics and to obtain rate constants for binding duplex DNA, we performed stopped-flow kinetic analysis for RPA and the RPA-XPA complex. We have previously published the stopped-flow kinetic analysis of RPA binding DNA using the intrinsic fluorescence properties of RPA and the quenching of fluorescence when RPA binds DNA. Here we demonstrate the effect of the XPA protein on the kinetic rates of RPA binding a duplex 41-bp cisplatin-damaged DNA (Fig. 4).

The quenching of the intrinsic tryptophan fluorescence of constant amounts of RPA or RPA-XPA complex was monitored over time at a variety of DNA concentrations. Control traces with buffer (used to zero the fluorescence) or DNA mixed with buffer were performed and resulted in minimal differences in fluorescence (data not shown). The traces presented in each figure represent the fluorescence above the buffer baseline value. Control traces obtained using RPA or the RPA-XPA complex mixed with buffer, which provides the initial fluorescence from which quenching was monitored, resulted in no change in fluorescence over time (data not shown). The in-
DNA Interactions of the RPA-XPA Complex

increase in the initial intrinsic fluorescence at time 0 for the RPA-XPA complex compared with RPA alone is due to the intrinsic fluorescence properties of the XPA protein (Fig. 4A). The intrinsic fluorescence of the XPA protein is not quenched when mixed with DNA similar to traces obtained with the buffer control (data not shown). In addition, when the anti-Xpress antibody was preincubated with the XPA protein, which results in enhanced DNA binding as judged by EMSA (Fig. 1), upon mixing with DNA, no change in intrinsic fluorescence was observed (data not shown). Each trace was fit to a double exponential decay (thick line), and the residual values for each fit are presented below the traces. The data were not consistent with a single exponential decay but were consistent with the slow phase of the biphasic reaction correlating with the denaturation of the duplex DNA followed by the binding of RPA to single-stranded DNA, consistent with our previously published data (22).

The observed rate of quenching ($k_{off}$) for the fast phase of the reaction was plotted versus DNA concentration for RPA (filled circles) and the RPA-XPA complex (open circles). The results revealed a linear relationship where the slope is equal to the $k_{on}$ and the y intercept equal to the $k_{off}$(Fig. 4B). The $k_{on}$ of RPA for the duplex cisplatin-damaged 41-mer DNA was $0.045 \pm 0.006 \text{ nM}^{-1} \text{s}^{-1}$, and the $k_{off}$ was $3.54 \pm 0.6 \text{ s}^{-1}$, consistent with the rate constants of RPA for a duplex 1,2d(GpG) cisplatin-damaged 30-mer DNA. The $k_{on}$ of the RPA-XPA complex for the duplex cisplatin-damaged 41-mer DNA substrate was $0.106 \pm 0.01 \text{ nM}^{-1} \text{s}^{-1}$ with a $k_{off}$ of $0.056 \pm 0.05 \text{ s}^{-1}$. These results reveal a 2–3-fold difference in the $k_{on}$ values, and even with the high error rate for the $k_{off}$ value there is at least a 3-fold difference in the $k_{eff}$ of RPA versus the RPA-XPA complex for the duplex cisplatin-damaged 41-mer. The $k_{on}$ of the RPA-XPA complex for the duplex undamaged 41-mer was $0.026 \pm 0.006 \text{ nM}^{-1} \text{s}^{-1}$ with a $k_{off}$ of $3.93 \pm 0.806 \text{ s}^{-1}$ (Table 1). These data are consistent with the XPA protein stabilizing RPA binding to duplex DNA and synergistically affecting the binding to duplex-damaged DNA.
XPA Has No Effect on RPA Single-strand DNA Binding

Kinetics—Considering the major role of RPA is single-strand DNA binding, stopped-flow kinetic analysis of RPA and the RPA-XPA complex binding single-stranded 40-mer DNA was performed (Fig. 5) to determine whether XPA had any effect on RPA single-strand DNA binding. The plot of \( k_{\text{obs}} \) versus DNA concentration for RPA (filled circles) yielded a \( k_{\text{on}} \) of 1.87 \( \pm 0.154 \) nM\(^{-1} \) s\(^{-1} \), and the \( k_{\text{off}} \) for the RPA-XPA complex (open circles) was 2.09 \( \pm 0.145 \) nM\(^{-1} \) s\(^{-1} \). The y intercept value \( (k_{\text{on}}) \) for RPA was 0.06 \( \pm 14.7 \) s\(^{-1} \), and the y intercept for the RPA-XPA complex resulted in a negative value with large errors, indicating a slow dissociation. The negative value for the y intercept \( (k_{\text{off}}) \) is a characteristic feature with high \( k_{\text{on}} \) values and a slow dissociation rate. A small change in the slope \( (k_{\text{on}}) \) can result in a dramatic change in the y intercept, and thus large errors associated with the \( k_{\text{off}} \) determinations. These results demonstrate minimal differences in the rate of association of RPA in the presence or absence of XPA when binding single-stranded DNA, and ultimately, XPA has no effect on RPA single-strand DNA binding kinetics. Interestingly, contaminating single-stranded DNA in mobility shift assays performed using duplex DNA results in less XPA protein super-shifted by the anti-Xpress antibody (data not shown). These results suggest that the XPA protein may dissociate from the RPA-ssDNA complex or RPA binding to single-stranded DNA results in a conformational change that disrupts the XPA interaction. The RPA-XPA complex remains intact, however, when the complex binds to duplex DNA, suggesting a different mode of duplex DNA binding for the RPA-XPA complex such that XPA can contact and stabilize the duplex DNA.

**DISCUSSION**

The process of eukaryotic NER has been well studied and reconstituted *in vitro*, but the initiation of the pathway including the recognition of the damaged DNA remains poorly understood (4, 5). Two proteins that are required for NER and that play an important role in the preincision NER complex are RPA and XPA (6, 14, 26). It has previously been shown that the RPA and XPA proteins interact in the absence of DNA and that both proteins are required before incision can occur (6, 12, 14), but little is known how the RPA-XPA interaction affects DNA binding and DNA damage recognition. In this study, we show a ternary complex of RPA and XPA bound to duplex cisplatin-damaged DNA, with the complex resulting in synergistic binding when compared with RPA or XPA alone. This suggests that the RPA and XPA proteins may form a complex prior to binding to DNA to enhance the specificity for damaged versus undamaged duplex DNA. Considering the low concentration of XPA relative to RPA, it is likely that a large portion of XPA is complexed with RPA, while a relatively small percentage of the cellular RPA is complexed with XPA. Clearly, it will be of interest to ascertain how the kinetics of the interaction of XPA with damaged DNA compare with the kinetics of the interaction of XPA with RPA bound to damaged DNA.

The RPA protein alone has previously been shown to preferentially bind duplex-damaged DNA compared with undamaged DNA, and the DNA binding activity correlates with the ability to denature the duplex DNA substrate (10, 11, 24). It has also been shown that RPA can unwind long stretches of DNA which is dependent on the reaction conditions (27, 28). In this study, the XPA protein inhibits the ability of the RPA protein to denature the duplex DNA substrate. This is an important function of the XPA protein so that the recruitment of the other repair factors can be localized to the damaged-DNA site. This would also maintain a defined system in which incision occurs −6 bases 3’ of the lesion and 22 bases 5’ , resulting in the release of a 24–32 nucleotide fragment (1, 2). Consistent with our previous model of RPA binding duplex DNA, the RPA-XPA complex binds duplex-damaged DNA followed by a defined denaturation such that RPA binds to the single-stranded DNA opposite the DNA adduct (24, 25). Evidence demonstrating that the XPA protein contacts both DNA strands enables the maintenance of a defined unwound duplex DNA around the DNA adduct (25). This would allow for the recruitment of the other repair factors including TFIIH, XPG (3') incision), and XPF-ERCC1 (5' incision) (1, 2, 29). The previously established protein interactions of the repair factors with RPA and XPA would position the XPG protein 3' of the DNA adduct and the XPF-ERCC1 protein 5' of the lesion (30–33). The positioning of RPA to the bottom undamaged DNA strand of the duplex favors the stimulation of DNA synthesis following damaged strand displacement (24, 34, 35). Although our data are consistent with RPA binding the undamaged strand of duplex-damaged DNA, RPA has been suggested to bind both strands of duplex-damaged DNA (36). The difference in binding in this case may be lesion specific.

### Table I

| DNA substrates        | \( k_{\text{on}} \) nM\(^{-1} \) s\(^{-1} \) | \( k_{\text{off}} \) s\(^{-1} \) | \( K_{\text{D}} \) nM |
|-----------------------|-------------------------------------------|-------------------------------|-----------------|
| RPA                   | 0.45 \( \pm 0.006 \)                      | 2.54 \( \pm 0.6 \)            | 78.7 \( \pm 16.45 \) |
| RPA-XPA               | 1.87 \( \pm 0.154 \)                      | 0.06 \( \pm 14.7 \)           | 0.032 \( \pm 7.87 \) |

\( \text{a.s.s.} \) 40-mer, single-stranded DNA.

\( \text{b. N.D.}, \) not determined.

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**FIG. 5.** Stopped-flow kinetic analysis of RPA and the RPA-XPA complex binding single-stranded 40-mer DNA. Kinetic traces were measured at a constant RPA concentration of 6.25 nM, in the absence or presence of 25 nM XPA (preincubated on ice), and varying DNA concentration from 62.5 to 125 nM. Each trace used an average of 10–12 measurements, and the kinetic traces were fit to a single exponential decay. The observed rate constants \( (k_{\text{obs}}) \) were then plotted versus DNA concentration and fit to a straight line. Data obtained for RPA binding (filled circles) and RPA-XPA complex binding (open circles) were compared to the single-stranded 40-mer DNA are presented. Each point represents the average of four separate experiments, and the error bars represent the S.D. The \( k_{\text{on}} \) values for RPA and the RPA-XPA complex are 1.87 nM\(^{-1} \) s\(^{-1} \) \( \pm 0.154 \) and 2.09 nM\(^{-1} \) s\(^{-1} \) \( \pm 0.145 \), respectively.
The kinetics of the binding reaction of the RPA-XPA complex is consistent with an increase in DNA damage specificity. The data are also consistent with the potential for shielding of cisplatin-damaged DNA by HMG-box proteins (37). These HMG-box proteins are highly abundant, and the A and B box domains of HMGB1 bind at rates to cisplatin-damaged duplex DNA substrates at near diffusion (38, 39). The kinetic data obtained with the RPA-XPA complex reveal a considerably slower rate of association, and considering the low cellular concentration of the complex, the potential for HMG-box proteins associating with the damaged sites before the NER machinery marks the site for repair is high.

Numerous caveats, however, remain to be investigated, not the least of which is the role of other proteins that can participate in the recognition of damaged DNA. The XPC-hHR23B protein complex also plays a role in the formation of the NER preincision complex (14, 15, 26). Recent data have demonstrated preferential binding of this complex to duplex-damaged DNA, and a series of repair kinetic reactions suggests that the XPC-hHR23B protein could, in some cases, be responsible for marking damaged sites and initiating repair (15, 40). More recently, in vivo analysis suggested that in the absence of XPA, XPC/hHR23B was still capable of relocating to the sites of DNA damage (21). Although these results suggest that XPC/hHR23B can potentially associate with damaged DNA, as assessed by immunofluorescence, the experiment does not reveal how the complex forms in the presence of XPA. Results were also presented suggesting that XPA was unable to associate with the sites of UV-induced DNA damage in XPC cells. However, the degree of relocation and association of XPA with the damaged sites observed in wild type cells was minimal, and therefore the results obtained monitoring XPA relocalization with the XPC cells are difficult to interpret (21). In addition to XPC-hHR23B and the XPA-RPA complex, the damaged DNA-binding protein DDB (heterodimer p127 and p48) also preferentially binds damaged DNA and is specifically involved in the repair of DNA adducts that fail to cause a large change in thermal stability of the duplex DNA (17, 41). Whether these protein factors play a role prior to RPA-XPA binding or whether there is a higher order recognition complex remains to be determined. It will be interesting to see how XPC-hHR23B and DDB affect the RPA-XPA complex association with duplex-damaged DNA.

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