Articles

Replication Protein A Interactions with DNA. III. Molecular Basis of Recognition of Damaged DNA†

Ye Lao,‡ Xavier V. Gomes,‡,§ Yingjie Ren,|| John-Stephen Taylor,|| and Marc S. Wold*,‡

Department of Biochemistry, University of Iowa College of Medicine, 51 Newton Road, Iowa City, Iowa 52242-1109, and Department of Chemistry, Washington University, St. Louis, Missouri 63130-4899

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ABSTRACT: Human replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein (subunits of 70, 32, and 14 kDa) that is required for cellular DNA metabolism. RPA has been reported to interact specifically with damaged double-stranded DNA and to participate in multiple steps of nucleotide excision repair (NER) including the damage recognition step. We have examined the mechanism of RPA binding to both single-stranded and double-stranded DNA (ssDNA and dsDNA, respectively) containing damage. We show that the affinity of RPA for damaged dsDNA correlated with disruption of the double helix by the damaged bases and required RPAs ssDNA-binding activity. We conclude that RPA is recognizing single-stranded character caused by the damaged nucleotides. We also show that RPA binds specifically to damaged ssDNA. The specificity of binding varies with the type of damage with RPA having up to a 60-fold preference for a pyrimidine(6−4)pyrimidone photoproduct. We show that this specific binding was absolutely dependent on the zinc-finger domain in the C-terminus of the 70-kDa subunit. The affinity of RPA for damaged ssDNA was 5 orders of magnitude higher than that of the damage recognition protein XPA (xeroderma pigmentosum group A protein). These findings suggest that RPA probably binds to both damaged and undamaged strands in the NER excision complex. RPA binding may be important for efficient excision of damaged DNA in NER.

The eukaryotic single-stranded DNA-binding protein, replication protein A is essential for multiple processes in cellular DNA metabolism including DNA replication, DNA repair, and recombination (reviewed in refs *1* and *2*). Human replication protein A (RPA)[1] is composed of subunits of 70, 32, and 14 kDa (RPA70, RPA32, and RPA14, respectively). RPA binds to single-stranded DNA (ssDNA) with low specificity and high affinity ($K_a = \sim 10^{10}$ M^{-1}) (*3*). Mutational and structural analysis have identified three domains of RPA that directly interact with single-stranded DNA: a weak ssDNA-binding domain in RPA32, a high-affinity central ssDNA-binding domain of RPA70 (which is composed of two copies of an oligonucleotide/oligosaccharide binding motif; OB fold), and the C-terminal domain of RPA70 (which contains a highly conserved zinc-finger motif) (Figure 1) (ref *4* and references therein). The central DNA-binding domain and the zinc-finger domain are both necessary and sufficient for high-affinity single-stranded binding activity (ref *4* and references therein). RPA has been shown to bind dsDNA and to promote helix destabilization (unwinding). The first two papers of this series (*4*, *5*) demonstrated that these DNA binding activities also depend on the central DNA-binding domain and zinc-finger domain of RPA70, and that double-stranded DNA binding is caused by RPA destabilizing the double helix and then binding to the resulting single-stranded regions in duplex DNA.

RPA has been shown to bind directly to damaged dsDNA. Initial studies demonstrated that RPA was able to bind to cisplatin-modified dsDNA (*6*). Subsequently it has been

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* To whom correspondence should be addressed: phone (319) 335-6784; Fax (319) 335-9570; Email marc-wold@uiowa.edu.

‡ University of Iowa College of Medicine.

§ Present address: Department of Biochemistry and Molecular Biophysics, Washington University, School of Medicine, St. Louis, MO 63110-1093.

|| Department of Chemistry, Washington, University.

[1] Abbreviations: RPA, human replication protein A; RPA70, RPA32, RPA14, 70-, 32-, and 14-kDa subunits of human replication protein A; RPA·70(Zn*), human replication protein A complex containing (C500S·C503S) 70-kDa subunit; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; NER, nucleotide excision repair; XPA, XPG, XPC, xeroderma pigmentosum complementation groups A, G, and C; TFIIH, transcription factor IIH; ERCC1/XPF, ERCC1/xeroderma pigmentosum complementation group F complex; DTT, dithiothreitol; GMSA, gel mobility shift assay; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; nt, nucleotide; bp, base pair; SSB, single stranded DNA-binding protein; (6−4) product, pyrimidine(6−4)pyrimidone product; CPD, cyclobutane pyrimidine dimer; c,s, *cis*,*syn* stereochemistry; t,s-I, *trans*,*syn*-I stereochemistry; t,s-II, *trans*,*syn*-II stereochemistry; Dewar product, Dewar valence isomer of the (6−4) product.

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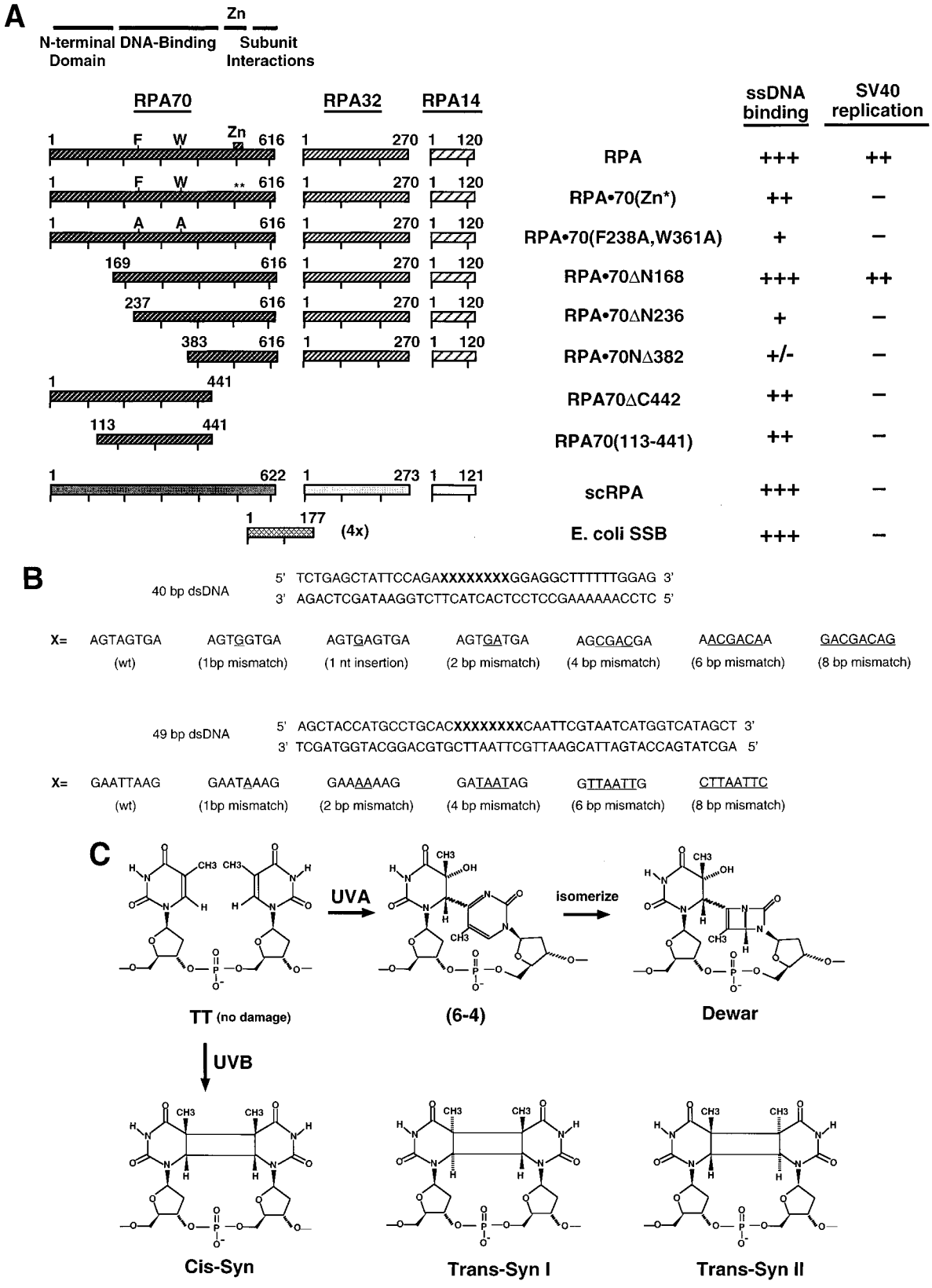

FIGURE 1: Proteins and DNAs used in these studies. (A) Schematic of wild-type and mutant forms of RPA used in these studies (abbreviations are indicated in parentheses): RPA, RPA·70(C500S,C503S) (RPA·70(Zn*)), RPA·70(F238A,W361A), RPA·70Δ1−168 (RPA·70ΔN168), RPA·70Δ1−236 (RPA·70ΔN236), RPA·70ΔN1−382 (RPA·70ΔN382), RPA70Δ442−616 (RPA70ΔC442), RPA70(113−441), yeast RPA (scRPA), and *E. coli* single-stranded DNA binding protein (SSB) (mutant forms described previously in refs *3*, *4*, and *59*). Tick marks indicate positions every 100 amino acids, and the position of the putative zinc-finger motif is indicated by the box labeled Zn. Approximate site of mutations in RPA·70(F238A,W361A) and RPA·70(Zn*) are indicated by As and stars, respectively. The single-stranded DNA binding and replication activities of these mutants are indicated. (B) Schematic of 40 bp dsDNA and 49 bp dsDNA containing different sizes of mismatched bubbles. The mismatched bases are underlined. Damage-containing 49-nucleotide DNAs were made by replacing the TT in the center of the GAATTAAG with a specific damaged adduct. (C) Structures of specific damaged adducts.

shown that RPA also binds preferentially to UV-damaged dsDNA and dsDNA modified by *N*-acetoxy-2-acetylaminofluorene (*7*, *8*). RPA has also been purified as a cisplatin-damaged dsDNA binding protein (*9*). Binding to double-stranded damaged DNA is less sensitive to salt and magnesium concentration than RPA binding to undamaged dsDNA (*9*).

Nucleotide excision repair (NER) is a primary pathway for repairing DNA damage caused by ultraviolet (UV) light and environmental mutagens. NER is a multistep process in which damage is first recognized and excised and the resulting gap filled in through a replicative process (reviewed in refs *10*−*13*). RPA is required for NER and is thought to be involved in all steps of this process. Recognition of sites of damage is critical for efficient NER. Initial studies suggested that damage was recognized by xeroderma pigmentosa group A protein (XPA) (*14*). This protein has no known enzymatic activity but binds to double-stranded DNA containing modified/damaged bases (*14*−*16*). Subsequently it was shown that XPA binding to damage was enhanced by RPA (*7*, *17*) and that nonhybridizing base pairs are involved in recognition and binding (*18*, *19*). Recent studies have also implicated XPC in damage recognition and initiation of NER (*20*, *21*); although in in vitro model reactions, excision occurred faster when XPA and RPA were the first damage recognition factors present (*21*). The proteins involved in damage recognition and repair interact with each other, complicating the elucidation of the mechanism of damage recognition. For example, RPA interacts with XPA, XPG, and ERCC1/XPF (*22*−*25*). RPA can also stimulate the endonuclease activities of both XPG and ERCC1/XPF (*25*, *26*). These interactions are thought to be essential for damage recognition, formation of the excision complex, and damage excision during NER(*21*, *27*; also reviewed in ref *56*).

To try to gain additional insights into the role of RPA in nucleotide excision repair and to understand the basis of RPA binding to damage-containing DNA, we have examined RPA binding to both double-stranded and single-stranded forms of DNA containing specific types of damage and compared those interactions with those of XPA. We show that RPA has an increased affinity for dsDNA containing DNA damage but that its affinity is lower than that of XPA. The affinity of RPA correlated with the change in melting temperature caused by a particular form of damage. We conclude that enhanced RPA binding primarily depends on the disruption of the duplex DNA caused by the damage. Surprisingly, we also found that RPA bound specifically to ssDNA containing damage. This specificity was dependent on the type of damage, with RPA having the highest affinity for pyrimidine-(6−4)pyrimidone photoproducts. Specificity for binding to damaged ssDNA was absolutely dependent on the zinc-finger domain of RPA70. RPA had a higher affinity than XPA for single-stranded damage containing DNA. These results suggest that RPA directly interacts with damaged ssDNA strand in the NER excision complex.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, polynucleotide kinase, and Klenow fragment were purchased from New England Biolabs and Life Technologies, Inc. [γ-^{32}P]ATP (4,500 Ci/mmol) and [α-^{32}P]dATP (3,000 Ci/mmol) were obtained from Amersham. *O*-Phenanthroline was obtained from Sigma. Oligonucleotides were purchased from Bio-Synthesis, Inc. *Escherichia coli* DH5α cells were from Life Technologies, Inc. *E. coli* expression strain BL21(DE3) was from W. Studier (*28*). *E. coli* single-stranded DNA-binding protein (SSB) was the generous gift of T. Lohman, Washington University, St. Louis.

HI buffer contains 30 mM HEPES (diluted from 1 M stock at pH 7.8), 1 mM dithiothreitol, 0.25 mM EDTA, 0.5% (w/v) inositol, and 0.01% (v/v) Nonidet-P40. HI was supplemented with different concentrations of salt as indicated. FBB buffer (1×) contains 30 mM HEPES (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.5% inositol, and 1 mM DTT. Tris−acetate/EDTA (TAE) gel buffer (1×) contained 40 mM Tris−acetate and 2 mM EDTA, pH 8.5, and Tris−borate/EDTA (TBE) gel buffer (1×) contained 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA (*29*).

Mutant Forms of RPA. Recombinant human RPA and the yeast homologue of RPA were expressed in BL21 (DE3) cells and purified as previously described (*30*, *31*). Deletion and mutational forms of RPA used in these studies are shown schematically in Figure 1. All mutant forms of RPA were expressed and purified as described previously (*4*).

DNA Substrates Containing Site-Specific Damage. Site-specifically damaged DNA was prepared as described previously (32). Briefly, d(AATTAA) was irradiated with 254 nm light, and the photoproducts were separated by C-18 HPLC. The major peak with an absorption maximum near 325 nm was collected and confirmed by NMR to be d(AA-[6−4]AA). The Dewar valence isomer of the (6−4) product, d(AA[Dewar]AA), was prepared in essentially quantitative yield by irradiation of d(AA[6−4]AA) with Pyrex and Mylar-filtered medium-pressure mercury lamp light. Oligonucleotides containing stereoisomers of the cyclobutane thymine dimer, d(CGAAT[c,s]TAAGC), d(AAT[t,s-I]TAA), and d(AAT[t,s-II]TAA), were prepared by solid-phase synthesis using TT photoproduct building blocks (*33*−*35*) and purified by anion-exchange and C-18 HPLC. The photoproduct-containing hexamers were individually enzymatically ligated to 18 nt and 25 nt oligonucleotides in the presence of a complementary 34 nt scaffold. The *cis*,*syn* dimer-containing decamer was similarly ligated to 16 nt and 23 nt oligonucleotides. Finally, the photoproduct-containing 49 nt oligonucleotides were isolated by preparative acrylamide gel electrophoresis. The sequence of the 49 residue oligonucleotide is shown in Figure 1B.

DNA Templates. The dsDNA fragment used as a probe for the UV-damage assay was generated by digesting pUC19 with *Hin*dIII and radiolabeled with [α-^{32}P]ATP. The DNA was subsequently digested with *Afl*III and the ∼400 bp fragment was isolated.

Single-stranded specific damaged 49 nt or parental 49 nt oligonucleotides used in ssDNA binding reactions were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (*29*). Labeled DNA was separated from free ATP with a Nensorb column following the manufacturer's protocol.

Double-stranded DNA containing either undamaged or specific damaged bases were made by individually annealing the labeled single-stranded 49 nt oligonucleotides (top strand, Figure 1B) with equal amounts of the complementary strand (lower strand in Figure 1B). Annealing reactions [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$] were incubated for 3 min at 95 °C and slowly cooled to room

temperature. The efficiency of annealing was examined by a 1× TBE 15% polyacrylamide gel followed by autoradiography. For all experiments, more than 95% of labeled DNA was in double-stranded form. Double-stranded fragments of 40 bp were made by the same protocol as for the 49 bp fragments. Sequences of the 40 bp fragments used are shown in Figure 1B, and the structures of the photoproducts used are shown in Figure 1C.

Gel Mobility Shift Assays. Gel mobility shift assays were performed as described previously with slight modifications (*36*). The indicated amounts of protein were incubated with 2 fmol of labeled DNA and 50 ng/μL BSA in 15 μL of either 1× FBB or the indicated buffer for 20 min at 25 °C. Binding reactions were brought to a final concentration of 4% glycerol and 0.01% bromophenol blue and electrophoresed on 1% agarose gel in 0.1× TAE at 10 V/cm for 1.5 h or as indicated. The gels were then dried on DE81 paper and radioactive bands were visualized by autoradiography. The radioactivity in each band was quantitated using a Packard instant imager. The resulting binding isotherms were analyzed by nonlinear least-squares fitting to the Langmuir binding equation with KaleidaGraph (Abelbeck Software) as described previously (*37*). In all cases the apparent binding constants shown is the average of multiple independent titrations.

RESULTS

RPA Binding to dsDNA Containing Specific Damaged Bases. RPA has been shown to bind preferentially to dsDNA containing damaged nucleotides (*6*−*9*). To confirm this observation, we examined binding of RPA to a 400 bp dsDNA fragment that had been irradiated with UV. RPA and labeled irradiated DNA were incubated, and complexes were separated by electrophoresis. The proportion of RPA−DNA complex increased in proportion to the UV exposure with greater than 80% of the DNA bound at UV dosages ≥ 11 kJ/m^2 (Figure 2B). Formation of this complex was dependent upon RPA concentration (Figure 2A). The midpoint of the titration with wild-type RPA occurred between 90 and 450 fmol of protein. This suggested that the binding constant of RPA for this population of damaged dsDNA is approximately 2 orders of magnitude lower than for ssDNA but at least 1 order of magnitude higher than for undamaged dsDNA (data not shown; see also below). We concluded that, under our assay conditions, RPA bound with high affinity to the UV-treated DNA. To elucidate the mechanism of this interaction, we next examined binding of RPA to defined dsDNA fragments containing specific photoproducts. The major types of DNA damage caused by UVB irradiation (280−320 nm) are cyclobutane dimers and (6−4) products (*38*−*41*). (6−4) photoproducts are not stable when exposed to 320 nm light and isomerize to their Dewar valence isomers (Figure 1C). Irradiation of duplex DNA mainly produces cyclobutane dimers of the *cis*,*syn* stereochemistry and only trace amount of the *trans*,*syn* stereochemistry (Figure 1C). ssDNA substrates, 49 nucleotides in length, were prepared in which the two central residues were either a pair of normal thymidines or a single dithymidine photoproduct (sequence shown in Figure 1B). These oligonucleotides were radiolabeled, annealed to a complementary sequence and used as substrates in gel mobility shift assays with RPA (data not shown). The amount of protein−DNA complex was quantitated, binding isotherms were generated, and apparent

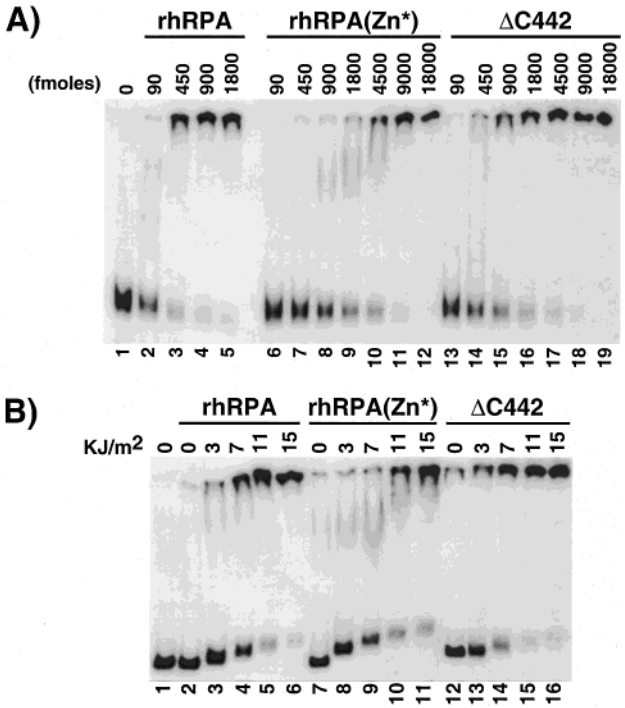

FIGURE 2: Binding of RPA to UV-damaged ssDNA. (A) RPA (450 fmol) was incubated with 2.3 fmol of either undamaged or UV-damaged (3, 7, 11, or 15 kJ/m) 400 bp fragment of dsDNA for 20 min at 25 °C. Lane 1 contained no added protein. (B) UV-damaged (11 kJ/m^2) 400 bp fragment of dsDNA (2.3 fmol) was incubated with increasing amounts of RPA. Assays were carried out and analyzed as described in Experimental Procedures. Electrophoresis was on 4% polyacrylamide gels in 0.5× TBE at 10 V/cm for 1.5 h.

Table 1: RPA Binding to 49 bp DsDNA with Specific Damage[a]

DNA	K_a (×10^{-6})	ratio	T_m (°C)
wt49	2.1 ± 0.5	1.0	49[b]
(6−4)	8.8 ± 2.5	4.2	21[b]
Dewar	8.9 ± 2.5	4.2	23[b]
cis,syn	2.8 ± 0.6	1.4	43[b]
trans,*syn*-I	4.5 ± 0.7	2.2	24
trans,*syn*-II	4.4 ± 0.9	2.1	24

[a] Binding assays were carried out in 1X FBB and analyzed as described in Experimental Procedures. Ratio K_a/K_a (wt). T_m is given for 15 μM dodecamer duplexes d(GAGTA*xy*ATGAG)−d(CTCAT-AATACTC) containing the damage indicated in 1 M NaCl. [b] From Jing et al. (42).

binding constants were determined (Table 1). We found that RPA bound dsDNA containing specific photoproducts with higher affinities than to undamaged dsDNA; binding to (6−4) and Dewar photoproducts was 4-fold higher than to undamaged DNA. Binding to *trans*,*syn* and *cis*,*syn* conformations was 2.2- and 1.4-fold higher than undamaged DNA, respectively.

Recently, we have shown that RPA binding to dsDNA is a manifestation of its ssDNA-binding activity (*5*). The current model for this process is that RPA initially destabilizes duplex DNA and then RPA forms a stable complex with the resulting ssDNA region (*5*). The initial formation of the complex between RPA and dsDNA seems to be slow relative to the later ssDNA binding. Furthermore, the affinity of RPA for dsDNA is 2−3 orders of magnitude lower than that for ssDNA DNA (*5*). This mechanism could also explain RPA binding to damaged dsDNA. Dithymidine photoproducts

Table 2: RPA Binding to Mismatches[a]

	40 bp		49 bp	
DNA	K_a ($\times 10^{-9}$)	T_m (°C)	K_a ($\times 10^{-9}$)	T_m (°C)
wild type	1.6 ± 0.3	63	1.7 ± 0.3	67
bubble (1 bp)	1.5 ± 0.4	61	1.5 ± 0.2	66
1 base insertion	1.5 ± 0.3	62	ND	
bubble (2 bp)	1.9 ± 0.5	60	1.4 ± 0.3	65
bubble (4 bp)	1.8 ± 0.7	60	1.4 ± 0.4	64
bubble (6 bp)	3.7 ± 1.0	54	3.29 ± 1.2	61
bubble (8 bp)	17.9 ± 6.5	50	11.2 ± 3.4	57

[a] Binding assays were carried out in HI buffer containing 30 mM KCl and analyzed as described in Experimental Procedures. T_m's determined as in ref *5*.

disrupt the duplex structure of DNA and reduce the melting temperature of the DNA (*42*). The resulting changes in melting temperature correlated with the affinity of RPA, with the hierarchy of binding (in order of decreasing affinity) being (6−4) and Dewar > ts-I and ts-II > cs > undamaged DNA. (6−4) and Dewar photoproducts caused large decreases in melting temperature and had the highest affinity for RPA (Table 1). *cis*,*syn* and *trans*,*syn* conformations have intermediate melting temperatures and intermediate affinities for RPA. The correlation between the log K_a and the melting temperature was not linear (data not shown), suggesting that factors other than duplex stability contribute to RPA recognition of damaged DNA.

To elucidate the role of unpaired bases in the binding of RPA to ssDNA, we examined RPA binding to double-stranded oligonucleotides containing various numbers of mismatched bases. We found that small mismatched bubbles (1−4 nt) caused small changes in the Tm (≤ 3 degrees) and had no effect on RPA binding (Table 2).[2] In contrast, mismatched bubbles of 6 or 8 nt caused decreases in T_m of 6−13 °C and caused the affinity for RPA to increase by as much as 10-fold (Table 2). Similar results were obtained for two independent series of oligonucleotides indicating that these observations are probably general effects caused by the mismatched nucleotides. In addition, these results are consistent with previous reports that RPA binds with high affinity to DNA duplexes containing 6 more unpaired nucleotides (*43*, *44*). We conclude that the single-stranded character introduced by damaged or mismatched bases contributed to the enhanced binding of RPA. To try to understand these interactions in more detail, we examined the binding of RPA to ssDNA containing damaged bases.

RPA Binding to Specific Damaged ssDNA. Binding of RPA to single-stranded 49-residue oligonucleotides containing either two unmodified thymidines or a single photoproduct was examined by gel mobility shift assay. Individual labeled ssDNAs were incubated with increasing amounts of RPA and then free DNA and protein−DNA complexes were separated by gel electrophoresis. In these assays a protein−DNA complex has a lower mobility than free DNA, and thus, binding is indicated by the appearance of low-mobility, radioactive complexes. RPA bound to all forms of single-stranded, damaged DNA (Figure 3). RPA has an occluded binding site size of 30 nt (*31*, *45*). Therefore the two bands with lower mobility observed correspond to singly and doubly liganded complexes (Figure 3). The amounts of free and RPA-bound ssDNA were quantitated, binding isotherms were generated, and apparent binding constants were determined (Table 3). The affinity of RPA for oligonucleotides containing thymidine photoproducts was in all cases higher than for the unmodified oligonucleotide. This difference ranged from a 4-fold increase for *trans*,*syn*-II to a 60-fold increase for (6−4) photoproduct. This binding specificity does not occur with all forms of damage. We found no increase in the affinity of RPA upon binding to oligonucleotides containing a cholesterol-modified base (data not shown). We conclude that RPA binds specifically to damaged ssDNA and that this binding is dependent on the form of modified base present, with RPA having the highest affinity for (6−4) photoproducts and their Dewar valence isomers.

The Conserved Zinc-Finger Motif of RPA70 Is Required for Specific Binding of RPA to Damaged ssDNA. RPA contains multiple domains involved in DNA binding (*1*, *2*, *4*). RPA70 is responsible for high-affinity binding to ssDNA and contains two domains that directly interact with ssDNA: a central ssDNA-binding domain and a highly conserved zinc-finger domain (*5*, *47*). We examined whether these domains also had a role in RPA binding to damaged ssDNA. Initially we examined the binding of RPA·70(Zn*) and RPA70ΔC442 to oligonucleotides containing specific photoproducts. These two forms of RPA have the conserved zinc finger either mutated or deleted, respectively (see Figure 1A). The intrinsic affinity of these two forms for ssDNA is approximately 1 order of magnitude lower than that of wild-type RPA (Table 4; see also ref *4*). Neither mutant form had a significant preference for binding to either (6−4) or cyclobutane dimer-containing ssDNA (Table 4). Previously, we have shown that treatment of RPA with *O*-phenanthroline causes ssDNA binding activity to decrease by the same amount as mutating the zinc-finger motif (*5*); these data were interpreted to indicate that chelation of a tightly bound metal inactivated the zinc-finger motif. Treatment with *O*-phenanthroline also eliminated the preference of wild-type RPA for the damaged ssDNA (Table 5). For example, the preference for ssDNA containing a (6−4) adduct went from a 30-fold higher affinity to only 2-fold (Table 5). These data indicate that the zinc-finger motif is required for preferential binding to damaged ssDNA and that a metal ion, probably zinc, is required for this process (shown schematically in Figure 4A).

We also examined the ability of other mutant forms of RPA to bind damaged ssDNA (Table 4). All forms of RPA containing an intact zinc-finger domain bound damaged ssDNA preferentially regardless of the overall affinity for ssDNA. RPA·70ΔN168, which has an intact central DNA-binding domain, has binding affinity similar to wild-type RPA. In contrast, RPA·70ΔN236, which has the central binding domain partially deleted, has a binding affinity more than 3 orders of magnitude lower than wild type (Table 4). Despite this large difference in intrinsic affinity, both RPA·70ΔN168 and RPA·70ΔN236 have intact zinc-finger domains and both bound damaged DNA preferentially. RPA70-(113−441) binds ssDNA with high affinity and consists of

[2] Note the experiments summarized in Tables 1 and 2 were carried out under different solution conditions and thus different apparent binding constants were determined. The binding assays in Table 1 were carried out under intermediate salt conditions (100 mM NaCl and 5 mM $MgCl_2$), while binding assays in Table 2 were carried out under low salt conditions (30 mM NaCl). We have shown previously that ionic strength affects RPA affinity for dsDNA by affecting the stability of the DNA duplex (*5*).

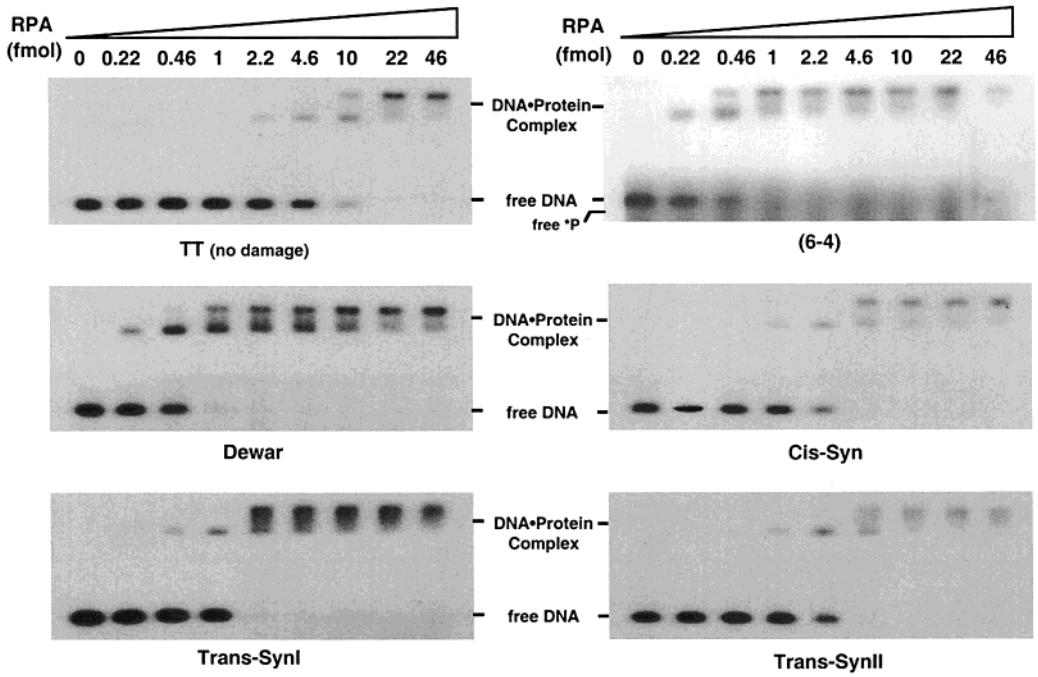

FIGURE 3: Binding of wild-type RPA to specific photodamaged ssDNA. Assays were carried out and analyzed as described in Experimental Procedures. The positions of the shifted complexes, free oligonucleotides, and free label are indicated. The type of specific damage contained in each oligonucleotide DNA is also indicated.

Table 3: Wild-Type RPA Binding to ssDNA with Specific Damage[a]

	K_a ($\times 10^{-8}$)	ratio
wt49	57 ± 17	1
(6−4)	3500 ± 1920	62
Dewar	1550 ± 270	27
cis,syn	320 ± 100	6
trans,syn-I	420 ± 230	8
trans,syn-II	230 ± 60	4

[a] Binding assays were carried out in 1× FBB and analyzed as described in Experimental Procedures. Ratio = K_a/K_a (wt).

only the central DNA-binding domain of RPA70. This mutant form does not contain the zinc-finger domain and had no specificity for damaged DNA (Table 4). These data confirm that the zinc-finger domain is necessary for specific binding to damaged DNA and suggest that it directly interacts with damaged bases. These studies do not distinguish whether the zinc-finger motif is directly interacting with the damaged bases or is required for maintaining the structure of this domain of RPA70. We also found that when the zinc finger is present without the central DNA-binding domain, as in RPA·70ΔN382, stable protein−DNA complexes were not observed with either parental ssDNA or specific damaged ssDNA (data not shown). We conclude that the central DNA-binding domain of RPA70 plays a predominant role in determining the overall affinity for DNA but does not contribute to specificity of binding to damage.

To determine whether other single-stranded DNA-binding proteins also preferentially bound to damaged oligonucleotides, we examined the binding of *E. coli* single-stranded DNA binding protein (SSB) and the yeast RPA homologue, scRPA. *E. coli* SSB does not contain a zinc-finger motif (*48*) and only showed a 3-fold higher affinity for the Dewar adduct containing ssDNA (Table 4). In contrast, scRPA, which contains a conserved zinc-finger motif, had a ≥22-fold higher affinity for Dewar photoproduct-containing ssDNA. This indicated that preferential binding for damaged dsDNA is a general property of RPA homologues but not of all single-stranded DNA binding proteins.

Taken together, our studies suggest that the specificity of RPA binding to damaged ssDNA arises because of the combined action of the nonspecific, high-affinity central DNA-binding domain and the low-affinity, damage-specific zinc-finger domain. The central binding domain directly interacts with 8 nt (*49*). Thus, we predicted that short oligonucleotides should show a weaker preference for binding to damage because they will be too short to interact fully with both domains (shown schematically in Figure 4B). Therefore, we tested our hypothesis by examining the binding of wild-type and mutant forms of RPA to a 12-residue oligonucleotide. RPA bound to the 12-residue oligonucleotide with lower affinity than observed with longer oligonucleotides (Table 5; see also ref *37*). No significant preference for binding a damage-containing 12-residue oligonucleotide was observed (Table 5). [The 2-fold preference observed with the 12-nt damaged oligonucleotide was equivalent to that seen with longer oligonucleotides and RPA·70(Zn*) or *O*-phenanthroline treated RPA.] Treatment with *O*-phenanthroline still caused a 10-fold decrease in affinity of RPA (Table 5). This suggests that a 12-nt oligonucleotide is long enough to partially interact with the zinc-finger domain. Alternatively, disruption of the zinc-finger motif by removal of associated metal could cause a change in the structure of the central binding domain. As was seen with longer oligonucleotides, RPA70ΔC442 showed no preference for binding damaged ssDNA and binding was insensitive to *O*-phenanthroline (Table 5). We also examined binding by RPA·70(F238A,W361A). This mutant form of RPA has two point mutations in the central binding domain of RPA70 that partially disrupt its structure and cause a reduction of approximately 3 orders of magnitude in the affinity for ssDNA (*4*). RPA·70(F238A,W361A) had a 10-fold higher

Table 4: Binding of Mutant Forms of RPA to ssDNA with Specific Damage[a]

RPA	undamaged K_a ($\times 10^{-8}$)	Dewar K_a ($\times 10^{-8}$)	ratio	*cis,syn* K_a ($\times 10^{-8}$)	ratio
RPA	57 ± 17	1550 ± 270	27	320 ± 100	6
RPA70Δ*C*442	6.9 ± 1.1	6.9 ± 0.9	1.0	6.3 ± 0.6	0.9
RPA·70(Zn*)	3.12 ± 0.7	4.9 ± 1.1	1.5	2.8 ± 0.8	0.9
RPA·70ΔN168	49 ± 5	≥3500	72[b]	440 ± 180	8.9
RPA·70ΔN236	0.0086 ± 0.0004	0.23 ± 0.04	27	0.11 ± 0.02	13
RPA70(113−441)	2.8 ± 0.3	5.4 ± 0.6	1.9	2.7 ± 0.3	1.0
scRPA	34 ± 5	≥770	22[b]	220 ± 50	6.2
E. coli SSB	13 ± 2	41 ± 9	3.0	15 ± 3	1.1

[a] Binding assays were carried out in 1× FBB and analyzed as described in Experimental Procedures. [b] Binding is under stoichiometric conditions, so binding constants were estimated from the concentration of RPA at half-saturation. Ratio = ratio of binding constants for damaged ssDNA/undamaged ssDNA.

Table 5: Binding of Wild-Type and Mutant Forms of RPA to Specific Damaged ssDNA ± Phenanthroline[a]

		49 nt oligonucleotide		
protein	phenanthroline	undamaged DNA K_a ($\times 10^{-8}$)	damaged DNA K_a ($\times 10^{-8}$)	ratio
wtRPA	−	52 ± 28	725 ± 250 (t,s-II)	14
wtRPA	+	1.3 ± 0.8	3.0 ± 1.6 (t,s-II)	2
wtRPA	−	39 ± 8	1200 ± 350 (6−4)	30
wtRPA	+	1.8 ± 0.7	3.5 ± 2.4 (6−4)	2

		12 nt oligonucleotide		
protein	phenanthroline	undamaged K_a ($\times 10^{-7}$)	(6−4) damaged K_a ($\times 10^{-7}$)	ratio
wtRPA	−	11 ± 1.5	24 ± 5	2.3
wtRPA	+	1.1 ± 0.2	1.3 ± 0.3	1.1
RPA70ΔC442	−	1.5 ± 0.2	2.4 ± 0.7	1.5
RPA70ΔC442	+	1.0 ± 0.1	1.6 ± 0.2	1.5
RPA·70(F238A,W361A)	−	0.12 ± 0.03	1.2 ± 0.1	9.7
RPA·70(F238A,W361A)	+	0.022 ± 0.015	0.027 ± 0.014	1.2

[a] Binding assays with 49 nt oligonucleotide were carried out in 1× FBB. Binding assays with 12 nt oligonucleotide [5′ GCGTA*TT*ATGCG 3′, with underlined TT being a (6−4) photoproduct] were carried out in 200 mM NaCl, 1 mM $MgCl_2$, 25 mM Hepes, 4% glycerol, 0.5 mM DTT, 0.01% NP-40 (*58*). The sequence of the analysis was carried out as described in Experimental Procedures. Ratio = ratio of binding constants for damaged ssDNA/undamaged ssDNA.

Table 6: Comparison of Binding Constants for XPA and RPA[a]

	K_a ($\times 10^{-7}$)		
DNA	RPA	XPA	XPA + RPA
ds49 bp	0.21 ± 0.05	1.2 ± 0.3	1.4 ± 0.4
ds49 bp, *cis,syn*	0.28 ± 0.06	3.0 ± 0.5	1.4 ± 0.3
ds49 bp, (6−4)	0.88 ± 0.25	3.3 ± 0.5	4.2 ± 0.4
ss49 nt	570 ± 170	0.46 ± 0.13	99 ± 26
ss49 nt, *cis,syn*	3200 ± 1000	0.55 ± 0.11	420 ± 110
ss49 nt, (6−4)	35,000 ± 19,000	0.18 ± 0.05	3000 ± 1100

[a] Binding assays were carried out in 1× FBB and analyzed as described in Experimental Procedures.

affinity for the (6−4) adduct-containing 12-nt oligonucleotide than for the undamaged 12-nt oligonucleotide (Table 5). This preference was sensitive to *O*-phenanthroline. The simplest explanation for these results is that when the central binding domain is partially disrupted, the short oligonucleotide is able to associate with the zinc-finger domain and preferential binding to damaged ssDNA is observed (shown schematically in Figure 4C). These data indicate that there is a competition between the central binding domain and the zinc-finger domain for ssDNA and that it is the interplay between these two sites that leads to the observed binding properties of RPA.

Binding of XPA and RPA to Damaged DNA. RPA and XPA are both involved in the damage recognition step of NER (*7*). Therefore, we also examined the binding of XPA protein to both single- and double-stranded forms of the damaged oligonucleotides. We found that XPA had an apparent binding constant for double-stranded, undamaged DNA of 1.2×10^7 (Table 6). This is approximately an order of magnitude higher than that of RPA under the same conditions. We observed additive binding to double-stranded DNA when RPA and XPA were mixed (Table 6). In contrast, XPA binds to ssDNA with an affinity 3 orders of magnitude lower than RPA and did not show any specificity for binding single-stranded, damaged oligonucleotides (Table 6 and data not shown). When binding of a mixture of RPA and XPA to single-stranded oligonucleotides was examined, the binding constants observed were slightly reduced from those of

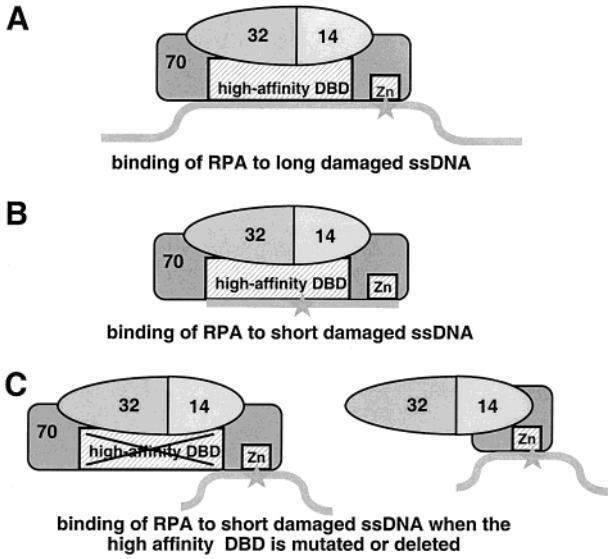

FIGURE 4: Models for RPA binding to (A) long damage-containing ssDNA and (B) short damage-containing ssDNA, and (C) binding of RPA in which the high-affinity DNA-binding domain has been mutated or deleted to short damage-containing ssDNA. Interactions with high-affinity DNA-binding (DBD) and zinc-finger (Zn) domains are shown. Subunits of RPA are indicated by their molecular mass. Star indicates position of damage.

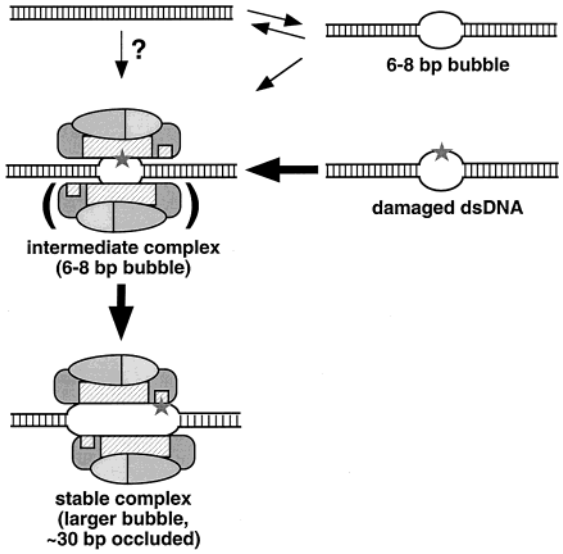

FIGURE 5: Model of RPA binding to damaged dsDNA. See text for details. Parts of RPA are as indicated in Figure 4. DNA is indicated by lines, and damage is indicated by the star.

RPA alone (Table 6). This suggests that RPA and XPA are competing for the damage and that binding of RPA is dominant. These data indicate that binding to damage is complicated. The relative affinities observed indicate that both RPA and XPA contribute to binding to dsDNA. In contrast, with ssDNA, RPA binding predominates with both undamaged and damaged strands.

DISCUSSION

In this paper we describe studies to define the mechanism of RPA binding to both single-stranded and double-stranded DNA containing damage. We have identified a new activity of RPA: specific binding to damaged ssDNA. We show that RPA binds preferentially to damaged ssDNA. The affinity of RPA varied depending upon the type of damage, with RPA having up to a 60-fold higher affinity for ssDNA containing pyrimidine(6−4)pyrimidone photoproducts. This specific binding depended upon multiple interactions between RPA and ssDNA. The zinc-finger domain of RPA70 is essential for specific binding, probably by directly interacting with the damaged nucleotides. In addition, the central, DNA-binding domain of RPA70 provides high-affinity, nonspecific interactions by interacting with ssDNA adjacent to the site of damage. A model of these interactions is shown schematically in Figure 4. This model is consistent with recent cross-linking studies showing that RPA70 predominantly interacts on the 5′ side of damage (*50*).

We have also quantitatively examined binding of RPA to dsDNA containing specific forms of damage. This analysis is more technically challenging than examining binding of RPA to ssDNA. Under most conditions, RPA destabilizes dsDNA, promoting its conversion to ssDNA (ref *5* and references within). In addition, the affinity of RPA for dsDNA is several orders of magnitude lower than that for ssDNA [K_a ssDNA $\sim 10^{10}$ M^{-1} vs K_a dsDNA $\sim 10^7$ M^{-1} (*5*)]. This means that when experiments are carried out under conditions that allow equilibrium binding of RPA to dsDNA, binding of RPA to ssDNA is stoichiometric. This precludes accurate determination of the affinity of RPA for ssDNA under the conditions used to examine dsDNA binding and makes it difficult to resolve contributions of RPA−ssDNA interactions under the conditions of dsDNA binding assays. Despite these difficulties, we were able to determine apparent binding constants for damaged DNA.

These studies indicate that interactions of RPA with damaged ssDNA differ from those with dsDNA. We showed that RPA binds to a short, damage-containing fragment of dsDNA with an affinity approximately 4-fold greater than that of the undamaged dsDNA. Binding to damaged dsDNA correlated with the extent of disruption of the duplex caused by the damage. On the basis of these studies and previous analysis of RPA binding to undamaged dsDNA (*5*), we conclude that binding of RPA to damaged dsDNA involves (at least) two steps: (1) an initial slow interaction step, which involves destabilization of the dsDNA and complex formation, and (2) a subsequent binding step in which RPA forms a stable complex with the newly formed ssDNA (shown schematically in Figure 5). This model is consistent with previous studies of RPA binding to damaged DNA (*6*−*9*) and with a recent study examining the role of nonhybridizing base pairs in damage recognition (*19*).

The function of the zinc-finger domain of RPA70 has been controversial (*4*, *5*, *47*, *51*−*53*); however, there is an emerging consensus that this domain directly interacts with ssDNA and contributes approximately 1 order of magnitude to the affinity of RPA (*5*, *47*). The zinc-finger domain is required for DNA replication (*4*, *53*) and base excision repair (*53*). We have shown previously that the zinc-finger domain is not essential for RPA destabilization of duplex DNA (*5*). We have also found that a mutant form of RPA with point mutations in the zinc-finger motif retained a preference for binding damaged dsDNA (Figure 2). We conclude that under the conditions used for these assays, the zinc-finger domain does not contribute significantly to RPA binding to damaged dsDNA. Instead, the disruption of the double helix caused by the damage seems to be a major source of specificity.

Presumably this is because the disrupted helix acts as a "nucleation site" for RPA during the slow initial binding event (Figure 5). Direct interactions with the zinc-finger domain appear to occur only with single-stranded DNA and thus occur after initial binding events that lead to unwinding the dsDNA. This model is consistent with the finding that a mutant form of RPA with a disrupted zinc-finger domain was functional in NER but was able to support only about 50% the level of excision as compared to wild-type RPA (*53*). These data indicate that, while not essential for NER, the zinc-finger domain is necessary for optimal excision of damage. We hypothesize that interactions between the zinc-finger domain and specific damage help align the excision nucleases and increase the efficiency of excision.

These studies provide new insights into the function of RPA during NER. RPA is required for the damage recognition step in NER (*7*, *54*, *55*). We present evidence that the role of RPA during damage recognition is to bind to the disruptions in the DNA duplex caused by damaged nucleotides. This activity requires RPA's ssDNA-binding activity and is closely related to RPA's dsDNA-binding and helix-destabilizing activities. The specificity of RPA for damaged dsDNA is modest; nevertheless, this interaction coupled with that of other damage binding proteins leads to the formation of a specific repair complex at the site of damage. Damage recognition also requires XPA (*14*−*16*) and XPC (*12*, *20*) and components of TFIIH (*12*). Individually, the specificities of these proteins for binding sites of damage are also modest (*12*, *14*, *16*). Therefore, it seems likely that high levels of specificity are achieved through the combined binding of multiple proteins with modest specificity (*12*, *19*) or through the binding of a "repair complex" containing several (or all) of these proteins (*56*).

After the initial damage recognition step, a protein complex forms at the site of damage and causes localized unwinding of the duplex and excision of the damage. RPA is also required for these processes. Most models propose that when the DNA is unwound, RPA binds to the undamaged strand while XPA binds to the damaged strand (*27*, *54*). Our studies show that this model is likely to be incorrect. The affinities of RPA for undamaged and damaged ssDNA are 3−5 orders of magnitude higher than those of XPA (Table 6; see also ref *19*). Therefore RPA should easily compete with XPA and bind to both unwound strands in the excision complex. Furthermore, RPA interacts with both the nucleases involved with excision, XPG and ERCC1-XPF (*7*, *25*). RPA can stimulate excision by XPG and ERCC1-XPF (*27*). This stimulation is enhanced in an orientation consistent with RPA binding to the undamaged strand; however, these studies did not rule out binding to both strands (*27*). We propose that RPA is bound to both strands in the excision complex and that specific protein−protein interactions in this complex are essential for efficient excision. The affinity of RPA is high enough that RPA should bind to both strands even in the absence of specific interactions with the damaged nucleotides. This could explain why the zinc-finger domain is not essential for NER (*53*). Mutant forms of RPA that bind ssDNA with high affinity but do not bind damage specifically [e.g., RPA·70(Zn*)] can still bind to both unwound strands in the excision complex; however, such mutant forms will not be positioned as precisely, leading to a reduced efficiency of excision. It is also possible that steric constraints or specific protein interactions may limit RPA binding in the damage excision complex. Additional studies will be needed to examine this possibility.

Both human and yeast RPA have conserved zinc-finger domains and bind specifically to damaged ssDNA. In contrast, *E. coli* SSB does not contain a zinc-finger motif and does not bind damaged ssDNA specifically. Functionally, both RPA homologues are essential for NER and involved in damage recognition, while *E. coli* SSB is not required for the equivalent pathway (uvrABC) in *E. coli*. These homologies suggest that there may be a general role for damage recognition in DNA repair processes. The single-stranded DNA-binding protein from phage T4, gene 32 protein, also contains a zinc finger and has been shown to bind damaged dsDNA (*57*). It will be interesting to determine whether gene 32 protein also binds damaged ssDNA specifically.

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