Replication Protein A Interactions with DNA. 2. Characterization of Double-Stranded DNA-Binding/Helix-Destabilization Activities and the Role of the Zinc-Finger Domain in DNA Interactions†

Ye Lao, Chang Geun Lee, and Marc S. Wold*

Department of Biochemistry, University of Iowa College of Medicine, 51 Newton Road, Iowa City, Iowa 52242-1109

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ABSTRACT: Human replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein that is composed of subunits of 70, 32, and 14 kDa. RPA is required for multiple processes in cellular DNA metabolism. RPA has been reported to (1) bind with high affinity to single-stranded DNA (ssDNA), (2) bind specifically to certain double-stranded DNA (dsDNA) sequences, and (3) have DNA helix-destabilizing ("unwinding") activity. We have characterized both dsDNA binding and helix destabilization. The affinity of RPA for dsDNA was lower than that of ssDNA and precisely correlated with the melting temperature of the DNA fragment. The rates of helix destabilization and dsDNA binding were similar, and both were slow relative to the rate of binding ssDNA. We have previously mapped the regions required for ssDNA binding [Walther et al. (1999) *Biochemistry 38*, 3963−3973]. Here, we show that both helix-destabilization and dsDNA-binding activities map to the central DNA-binding domain of the 70-kDa subunit and that other domains of RPA are needed for optimal activity. We conclude that all types of RPA binding are manifestations of RPA ssDNA-binding activity and that dsDNA binding occurs when RPA destabilizes a region of dsDNA and binds to the resulting ssDNA. The 70-kDa subunit of all RPA homologues contains a highly conserved putative (C-X_2-C-X_{13}-C-X_2-C) zinc finger. This motif directly interacts with DNA and contributes to dsDNA-binding/unwinding activity. Evidence is presented that a metal ion is required for the function of the zinc-finger motif.

Human replication protein A (RPA)[1] is a heterotrimeric complex with three subunits of 70, 32, and 14 kDa. RPA was initially identified as a protein that was essential for SV40 DNA replication (1−3). Subsequently, RPA has been shown to be required for multiple processes in cellular DNA metabolism including DNA replication, DNA repair, and recombination (reviewed in *4*). Heterotrimeric RPA homologues have been found in all eukaryotic cells examined (*4*, *5*). These proteins are highly conserved, and all seem to be required for DNA metabolism. RPA interacts with proteins involved in DNA replication, DNA repair, recombination, transcription, and cell regulation (reviewed in *4*; see also *6*−*9*). The role of these protein interactions is not well understood, but it is thought that specific RPA−protein interactions are needed for RPA function. RPA is also known to be phosphorylated during cellular DNA metabolism (reviewed in *4*).

RPA binds to ssDNA with low specificity and high affinity. RPA has an association constant of approximately 10^{10} M^{-1} (*10*−*12*). RPA also has been shown to bind ssDNA with low cooperativity ($\omega = 10$), and has a preference for binding polypyrimidine tracts (*13*, *14*). RPA has an occluded binding site size of 30 nucleotides (*10*, *15*), although a less stable binding mode has been observed in some experiments after chemical cross-linking(*16*, *17*). The single-stranded DNA-binding properties of RPA homologues are generally similar [reviewed in (*4*)], although it has been recently shown that there are significant differences between the binding of human RPA and the RPA homologue from *Saccharomyces cerevisiae* (scRPA) (*15*).

RPA has also been reported to have other DNA-binding activities. RPA was observed to bind specifically to certain double-stranded DNA (dsDNA) sequences in yeast and sea urchins and to be part of a complex that interacts with the metallothionein promoter in human cells (*18*−*21*). These sequences have been postulated to be involved in the regulation of gene expression (*18*−*21*). However, there does not appear to be a common consensus sequence for RPA binding because different sequences were found to bind RPA in each of these systems (discussed in *4*). RPA has also been found to have an intrinsic "DNA-unwinding" activity (*22*, *23*). This activity does not require ATP hydrolysis and is most efficient at low ionic strength. Thus, RPA "unwinding" is not caused by a helicase-like activity but instead is a helix-destabilizing activity (*22*−*24*). While all ssDNA-binding proteins can destabilize dsDNA, RPA seems to be more efficient at destabilizing dsDNA than other ssDNA-binding

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

[1] Abbreviations: RPA, human replication protein A; RPA70, 70-kDa subunit of RPA; RPA32, 32-kDa subunit of RPA RPA14, 14-kDa subunit of RPA; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DTT, dithiothreitol; GMSA, gel mobility shift assay; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; nt, nucleotide; bp, base pair; SSB, single-stranded DNA-binding protein.

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proteins (*22*, *23*). DNA fragments up to 1000 bp can be unwound by RPA (*22*, *23*). RPA is also known to stimulate the T antigen-dependent unwinding of the SV40 origin (*2*, *4*, *25*−*28*). This reaction is one of the first steps in the initiation of SV40 DNA replication and involves RPA stabilization of ssDNA generated at the SV40 origin by T antigen helicase activity (reviewed in *4*). RPA's role in this reaction is nonspecific in that other ssDNA binding proteins can also stimulate T antigen-dependent unwinding (*4*). Finally, it has been shown that RPA binds preferentially to dsDNA containing damaged bases (*29*−*32*). This binding to damaged DNA may be important in the recognition step of nucleotide excision repair in eukaryotic cells (*4*, *29*, *30*,-*33*). The only DNA-binding activity of RPA that has been studied in detail is the ssDNA-binding activity (reviewed in *4*). It is currently not known how these various DNA-binding activities are related or the degree to which RPA participates in these multiple types of interactions with the DNA in vivo.

Deletion and mutational analyses of RPA have identified several functional domains in the three subunits (*11*, *12*, *34*−*40*). The 70-kDa subunit is composed of four domains. The N-terminal 168 amino acids are principally involved in RPA−protein interactions. The central domain (residues ∼170 through 441) is composed of two repeats of a DNA-binding motif termed the oligonucleotide/oligosaccharide-binding (OB) fold and has ssDNA-binding activity and participates in some RPA−protein interactions. Residues ∼481 through ∼503 contain a highly conserved putative zinc-finger motif which is required for optimal ssDNA-binding activity (*12*). Studies of this domain have been contradictory (*36*, *37*, *41*, *42*); however, there seems to be a growing consensus that mutation of the zinc-finger motif inactivates DNA replication activity and decreases ssDNA binding (*12*, *36*, *41*). The C-terminal region of the 70-kDa subunit is required for interactions with the other RPA subunits. The 32-kDa subunit is composed of 3 domains: an N-terminal domain of approximately 35 residues, which is phosphorylated and involved in regulation of RPA function (*43*−*45*), a central domain, which participates in subunit interactions and has weak ssDNA-binding activity (*15*, *46*−*49*); and a C-terminal domain that participates in RPA−protein interactions (*12*, *33*, *42*, *50*−*52*). Little is known about the 14-kDa subunit except that it seems to be essential for RPA complex assembly (*53*, *54*).

In the previous paper, we have used a series of mutant forms of RPA to elucidate the role of the central DNA-binding and the putative zinc-finger domains in RPA interactions with ssDNA (*12*). In this paper, we extend these studies by carrying out a systematic analysis of RPA dsDNA-binding and helix-destabilizing activities. We show that all DNA binding activities map to the central ssDNA-binding domain of RPA70. We find that dsDNA binding correlates with the melting temperature of the dsDNA. Time courses show that the rates of RPA binding to dsDNA and helix destabilization are similar and that both are much slower than ssDNA binding. We conclude that dsDNA-binding and helix-destabilizing activities are tightly linked and that both are manifestations of the ssDNA-binding activity of RPA. The zinc-finger domain is essential for efficient dsDNA binding/unwinding. We also show that the zinc-finger motif directly interacts with DNA and that this interaction requires a metal ion.

MATERIALS AND METHODS

Materials. Polynucleotide kinase was purchased from New England BioLabs and Life Technologies, Inc. [γ-^{32}P]ATP (4500 Ci/mmol) and [α-^{32}P]dATP (3000 Ci/mmol) were obtained from Amersham. Oligonucleotides were synthesized by the DNA core facility at the University of Iowa. Oligodeoxythymidine [$(dT)_{30}$] was purchased from Bio-Synthesis, Inc.

HI buffer contains 30 mM HEPES (diluted from 1 M stock at pH 7.8), 1 mM dithiothreitol (DTT), 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. 1× FBB buffer: 30 mM HEPES (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.5% inositol, and 1 mM DTT. 1× Tris acetate/EDTA (TAE) gel buffer contained 40 mM Tris−acetate and 2 mM EDTA, pH 8.5, and 1× Tris borate/EDTA (TBE) gel buffer contained 89 mM Tris base, 89 mM boric acid, 2 mM EDTA (*55*).

A schematic of all of the forms of RPA used in these studies is shown in Figure 1. Generation and purification of wild-type and mutant forms of RPA have been described previously (*11*, *12*, *53*).

DNA Templates and Manipulation. The 40 nt dsDNA fragment used in dsDNA-binding and helix-destabilizing reactions was made by labeling an oligonucleotide from the SV40 origin (SV40 top: 5′-CTCCAAAAAAGCCTCCT-CACTACTTCTGGAATAGCTCAGA-3′) with [γ-^{32}P]ATP by T4 polynucleotide kinase (*55*). The labeled DNA was separated from free ATP with a Nensorb column following the manufacturer's protocol and annealed to an equal amount of the complementary sequence (SV40 bottom: 5′-TCTGA-GCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAG-3′). The 40 nt fragment with an 8 nt single-stranded "bubble" was made by annealing SV40 top to SV40 bottomB (5′-TCTGAGCTATTCCAGAGACGACAGGGAGGCTTTTT-TGGAG-3′) where underlines indicate bases that have been changed to be noncomplementary. Annealing reactions [10 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 50 mM NaCl] were incubated at 95 °C for 3 min and slowly cooled to room temperature over 2 h. Efficiency of annealing was determined by analyzing DNA by 15% polyacrylamide gel electrophoresis (1× TBE) followed by autoradiography. For all experiments, more than 95% of labeled DNA was in double-stranded form.

Restriction endonucleases and Klenow were used according to manufacturers' recommendations.

Gel Mobility Shift Assays. Gel mobility shift assays were performed as described previously with slight modifications (*13*). Binding reactions with ssDNA templates were carried out in 1× FBB, and binding reactions with dsDNA templates were carried out in HI buffer containing 30 mM NaCl or in the indicated buffer. Fifteen microliter reactions containing the indicated amount of wild-type or mutant form of RPA, 2 fmol (or indicated amount) of DNA, and 50 ng/μL BSA were incubated at 25 °C for 20 min. Binding reactions were then brought to a final concentration of 4% glycerol and 0.01% bromphenol blue and separated on a 1% agarose gel in 0.1× TAE at 100 V/cm for 1.5 h. The gels were dried on DE81 paper, and the radioactive bands were visualized by autoradiography. The radioactivity in each band was quantitated using a Packard Instant Imager. The data were

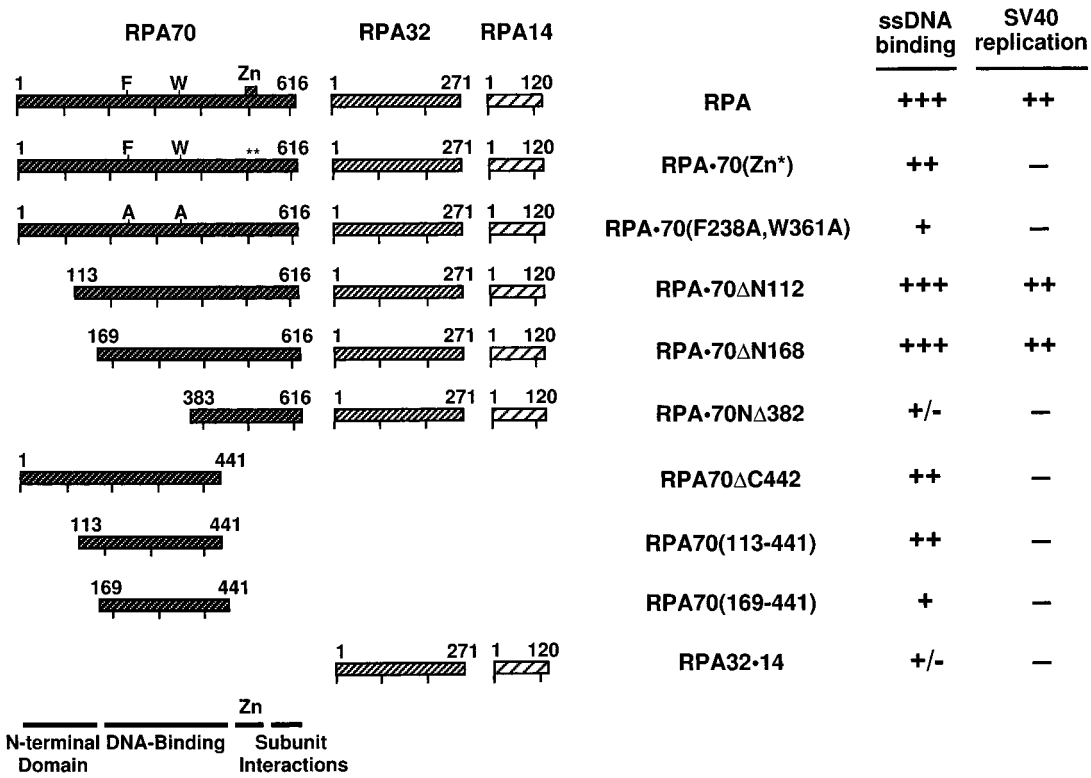

FIGURE 1: Mutant forms of RPA used in these studies. All forms of RPA used in this study are shown. Beginning and ending amino acids of each mutant are indicated. Ticks indicate positions every 100 amino acids, and the position of the zinc-finger motif is indicated by a box (Zn). Lines below the schematic indicate the location of functional domains of RPA70. The activities of each of the mutants in relation to wild-type RPA determined previously (*11, 12, 15*) are shown to the right. ssDNA-binding activity determined with $(dT)_{30}$: (+++) wild-type binding; (++) binding reduced by an order of magnitude; (+) binding reduced by 2 or more orders of magnitude; (±) binding reduced by more than 5 orders of magnitude. Replication activity: (++) activity similar to wild type; (−) no activity. The following RPA mutants were used in these studies (abbreviations in parentheses): wild-type RPA (RPA), RPA·70(C500S,C503S) (RPA·70(Zn*)), RPA·70 (F238A,W361A), RPA·70Δ1−112 (RPA·70ΔN112), RPA·70Δ1−168 (RPA·70ΔN168), RPA·70Δ1−382 (RPA·70ΔN382), RPA70ΔC442−616 (RPA70ΔC442), RPA70(113−441), RPA70(169−441), and RPA32·14.

analyzed by nonlinear least-squares fitting to the Langmuir binding equation using Kaleidagraph (Abelbeck Software) as described previously (*10*).

Helix-Destabilization Assay. Helix-destabilization assays were performed as described for gel mobility shift assays using dsDNA except that reactions were terminated by adding SDS to a final concentration of 0.2% (to disrupt RPA−DNA complexes) and separated on a 15% polyacrylamide gel in 1× TBE at 100 V/cm for 3 h.

Melting Temperature Determination. Thermal denaturation of oligonucleotide duplexes was followed by observing the hyperchromicity at 260 nm using a Gilford Model Response II thermal spectrophotometer. 1.0 nmol of oligonucleotide was incubated with 1.0 nmol of the complementary oligonucleotide in 350 μL of HI buffer containing 30 mM NaCl or the indicated buffer. Samples were heated from 25 to 80 °C over 1 h with the absorbance measured every 1 °C. Melting temperatures were obtained from the maximum value of the first-derivative plots of absorbance vs temperature. Melting temperatures were accurate to 0.5 °C.

RESULTS

RPA Interactions with dsDNA. To systematically examine RPA binding to dsDNA, we examined RPA binding to a 40 nt fragment derived from the SV40 origin of replication. In these assays, either the single-stranded or the double-stranded form of the oligonucleotide was radiolabeled and incubated with increasing amounts of RPA, and the products were separated on an agarose gel. RPA−DNA complexes were detected by changes in mobility of the radiolabeled DNA fragment (Figure 2A, top panel). The amounts of free DNA and DNA−protein complex were quantitated, and an apparent association constant was determined from the resulting binding isotherms. When binding was examined at near-physiological ionic conditions (100 mM KCl, 5 mM $MgCl_2$), RPA bound the single-stranded oligonucleotide with an affinity of 2.7×10^{10} M^{-1} (Table 1) and bound the double-stranded oligonucleotide with an affinity almost 3 orders of magnitude lower (data not shown; see also Table 2, below). These results are consistent with previous analyses of RPA binding to dsDNA (*13, 56, 57*). The affinity of RPA for dsDNA increased as the ionic strength was decreased. The binding constant for the 40 nt double-stranded fragment was 1.6×10^9 M^{-1} at 30 mM KCl (Table 1). We also examined RPA binding to a variety of other dsDNA sequences under low-salt conditions. RPA was able to bind to all DNA fragments examined regardless of sequence or length (data not shown). DNA ends are not required for binding because similar affinities were observed with circular plasmids and long linear DNA (data not shown). The apparent binding constants determined for different sequences were found to vary over approximately an order of magnitude, although several DNA fragments were found that bound RPA very poorly (data not shown).

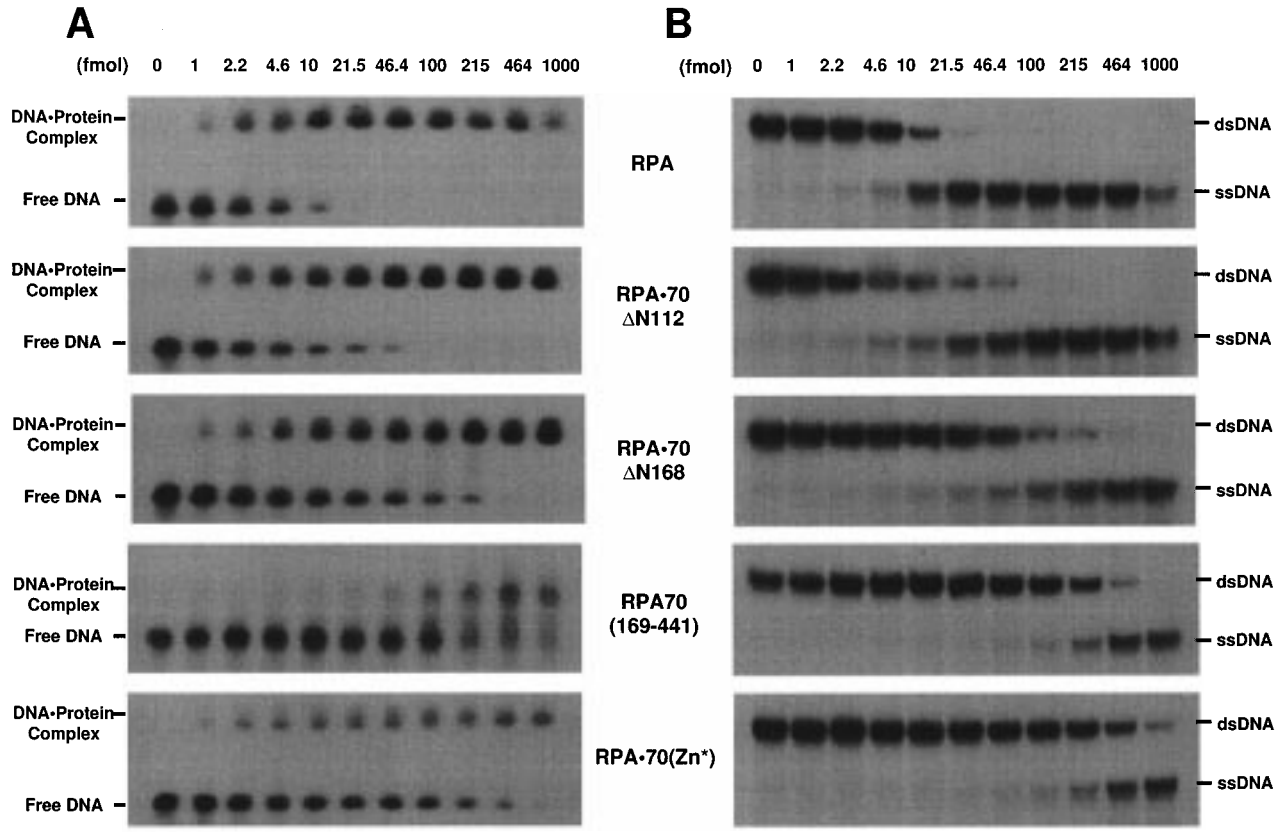

FIGURE 2: dsDNA-binding (A) and helix-destabilizing (B) activities of wild-type and selected mutant forms of RPA. The indicated amounts of wild-type and mutant forms of RPA protein were incubated with 2 fmol of radiolabeled double-stranded 40 nt fragment in HI buffer with 30 mM KCl and 50 ng/μL BSA for 25 min at 25 °C. Reactions were then terminated and separated by electrophoresis as described under Materials and Methods for dsDNA binding (A) and helix destabilization (B). (A) The positions of the DNA−protein complex and free DNA are indicated. (B) The positions of ss- and dsDNA are indicated.

Table 1: DNA-Binding Activities of Forms of RPA[a]

	K_a(40−mer) ($\times 10^{-8}$ M^{-1})	K_a(dsDNA)($\times 10^{-8}$ M^{-1})	helix destabilizing ($K_{a,app} \times 10^{-8}$ M^{-1})
RPA	270 ± 140 (1) S	16 ± 3 (1)	7.8 ± 1.2 (1)
RPA70ΔC442	48 ± 8 (0.18)	4.1 ± 1.0 (0.25)	1.7 ± 0.8 (0.22)
RPA•70ΔN112	260 ± 160(0.96) S	16 ± 4.4 (0.97)	6.5 ± 1.2 (0.83)
RPA•70ΔN168	280 ± 180 (1.0) S	2.5 ± 1.0 (0.15)	1.2 ± 0.1 (0.16)
RPA•70ΔN382	NS	nd	nd
RPA•70(F238A,W361A)	0.53 ± 0.05 (0.002)	0.03 ± 0.005 (0.002)	0.016 ± 0.006 (0.002)
RPA70(113−441)	46 ± 11 (0.17)	4.1 ± 1.5 (0.25)	2.0 ± 0.9 (0.25)
RPA70(169−441)	nd	0.30 ± 0.07 (0.02)	0.22 ± 0.07 (0.03)
RPA•70(Zn*)	24 ± 8 (0.09)	0.48 ± 0.19 (0.03)	0.19 ± 0.03 (0.02)

[a] Apparent DNA-binding constants and helix-destabilizing activities for mutant forms of RPA. Numbers in parentheses indicate ratio of binding constant to that of wild-type RPA. The average association constant from multiple independent titrations is shown; errors were calculated by determining the square root of the average of the squares of the error determined by nonlinear least-squares fitting. S, stoichiometric or near-stoichiometric binding conditions; NS, it was not possible to saturate binding; nd, not determined.

Table 2: Dependence of dsDNA-Binding Activity of RPA on Solution Conditions

DNA	buffer	salt	K_a ($\times 10^7$)	melting temp (°C)
40-mer	HI	30 mM KCl	170 ± 20	64 ± 0.5
40-mer	HI	60 mM KCl	30 ± 6.2	67 ± 0.5
40-mer	HI	100 mM KCl	4.3 ± 1.1	71 ± 0.5
40-mer	FBB(−Mg)	100 mM KCl	4.2 ± 0.7	71 ± 0.5
40-mer	FBB	100 mM KCl, 5 mM $MgCl_2$	0.56 ± 0.26	75 ± 0.5

We have shown that the central DNA-binding domain of RPA (residues ~170−445 of RPA70) is necessary for ssDNA binding and that sequences immediately adjacent to this domain also contribute to RPA-binding activity (*12*). To determine if the same regions of RPA were needed for dsDNA-binding activity, a series of binding assays were carried out using various mutant forms of RPA (shown schematically in Figure 1). The binding constants were determined for each mutant form of RPA using the single-stranded form of 40 residue oligonucleotide for reference (summarized in Table 1). The binding constants obtained for the single-stranded oligonucleotide were similar to those determined previously with a 30 nt oligonucleotide composed of deoxythymidine (summarized in Figure 1; *12*).

Figure 2A shows representative titrations of dsDNA with several mutant forms of RPA. These data were then quantitated, binding isotherms generated, and apparent association constants determined (Table 1). In general, we

found that ssDNA- and dsDNA-binding activities correlated for the different mutant forms of RPA. Under these conditions for ssDNA and dsDNA binding, the appearent binding constants for dsDNA were usually $\sim^1/_{10}$th those for ssDNA. For example, RPA70ΔC442, a polypeptide that contains residues 1−441 of RPA70 (Figure 1), has affinities for ssDNA and dsDNA that are 4.8×10^9 and 4.1×10^8 M^{-1}, respectively, and both are approximately one-fifth the corresponding affinity of wild-type RPA (Table 1). The two exceptions to this generalization were RPA·70ΔN168 and RPA·70(Zn*), which both had a greater decrease in dsDNA binding than in ssDNA binding (Table 1). This decrease is especially pronounced in RPA·70ΔN168 which has wild-type affinity for ssDNA (see also *11*) but an affinity for dsDNA one-sixth that of wild-type RPA (Figure 2A and Table 1).

The isolated central DNA-binding domain of RPA70 [RPA70(169−441)] was sufficient to give dsDNA-binding activity (Figure 2A), but its affinity was only $^1/_{50}$th that of wild-type RPA (Table 1). When the DNA-binding domain was mutated [RPA·70(F238A,W361A)], dsDNA-binding activity was essentially eliminated (Table 1). We conclude that the DNA-binding domain in RPA is both necessary and sufficient for dsDNA-binding activity but that sequences outside the minimal binding domain contribute significantly to optimal dsDNA-binding activity. Residues 112−168 of RPA70 and the putative zinc finger near residue 500 of RPA70 both contributed to dsDNA binding [compare K_a of RPA70(169−441) to RPA70(112−441) and RPA·70(Zn*) to RPA].

Helix-Destabilizing Activities of RPA. We also tested the mutant forms of RPA shown in Figure 1 for helix-destabilizing activity. Helix destabilization was monitored in a modified RPA-binding assay. The 40 bp dsDNA was incubated with different forms of RPA and protein−DNA complexes dissociated with SDS, and the amounts of ssDNA and dsDNA were quantitated after separation on a 15% polyacrylamide gel. Figure 2B shows assays examining several mutant forms of RPA. Approximately 10 fmol of RPA or RPA·70ΔN112 was needed to convert 50% of the dsDNA to single-stranded form. Substantially larger amounts of several mutant forms of RPA [e.g., RPA70(169−441) and RPA·70(Zn*)] were needed to obtain similar destabilization (Figure 2B).

We quantitated the amounts of ssDNA and dsDNA and then fitted the resulting isotherm to the Langmuir binding equation to obtain apparent binding constants for the destablization reaction (Table 1). Although this reaction may not be a simple bimolecular reaction, helix destabilization requires direct interactions between RPA and ssDNA, and the apparent binding constant determined provides a quantitative measure of the activity in this assay. We found that the relative activity of the mutant forms of RPA in the helix-destabilizing assay was identical to their relative activity in the dsDNA-binding assay (compare ratios shown in Table 1). Polypeptides containing the DNA-binding domain of RPA [e.g., RPA70(113−441) and RPA70(168−441)] had significant helix-destabilizing activity, indicating that the DNA-binding domain of RPA70 is sufficient for helix destabilization. However, as observed in the dsDNA-binding assays, deletion of residues between 112 and 168 of RPA70 or mutation of the zinc-finger domain caused a significant reduction in helix-destabilizing activity [compare RPA70(169−441) to RPA70(112−441) and RPA·70(Zn*) to RPA; Table 1]. The similarity in the relative activities of the mutant forms of RPA in these three DNA-binding assays suggested that all three activities are related.

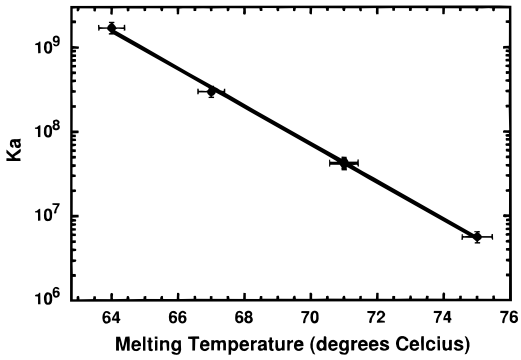

FIGURE 3: dsDNA binding versus DNA melting temperature. RPA-binding constants determined for the double-stranded 40 nt fragment were plotted against the experimentally determined melting temperature of the fragment under different solution conditions. Data are listed in Table 2. The best-fit line is shown.

Relationship between dsDNA-Binding and Helix-Destabilizing Activities. To elucidate the link between dsDNA binding and helix destabilization, the dependence of both activities on solution conditions was determined. Previous studies have indicated that helix destabilization is strongly inhibited by elevated levels of magnesium or monovalent cations (*22*, *23*, *58*). We confirmed these observations (data not shown). We also found that the affinity of RPA for dsDNA decreased as ionic strength increased, with magnesium ions being more inhibitory than monovalent cations (Table 2). Decreasing the KCl concentration from 100 to 30 mM increased the apparent affinity of RPA for dsDNA 40-fold (Table 2), and increasing the concentration of $MgCl_2$ from 0 to 5 mM caused the affinity of RPA to decrease by almost an order of magnitude (Table 2). The combination of 100 mM KCl and 5 mM $MgCl_2$ decreased the affinity for dsDNA by almost 3 orders of magnitude (Table 2). Similar results were observed with a variety of DNA fragments under multiple reaction conditions (data not shown). The melting temperature of the DNA fragment was determined for each solution condition shown in Table 2. We found that the apparent binding constant for dsDNA was linearly correlated with the observed melting temperature of the DNA on a semilog plot (Figure 3). This demonstrated that the free energy of RPA binding to dsDNA is proportional to the melting temperature and that dsDNA binding is directly dependent on the stability of the DNA helix. This conclusion is further supported by the fact that binding of RPA to dsDNA increases when a mismatched bubble exists in the DNA duplex, and the binding constant increases as the bubble size increases (Y. Lao, unpublished data).

To further compare the relationship between ssDNA-binding, dsDNA-binding, and helix-destabilizing activities of RPA, time courses were carried out (Figure 4). Binding of RPA to ssDNA is very rapid with most of the ssDNA being bound before the first time point (0.5 min; Figure 4E). In contrast, both dsDNA-binding and helix-destabilization reactions had the same kinetics and were much slower with saturation occurring between 5 and 10 min (Figure 4A,B,F). This suggests that these activities are directly linked. To test

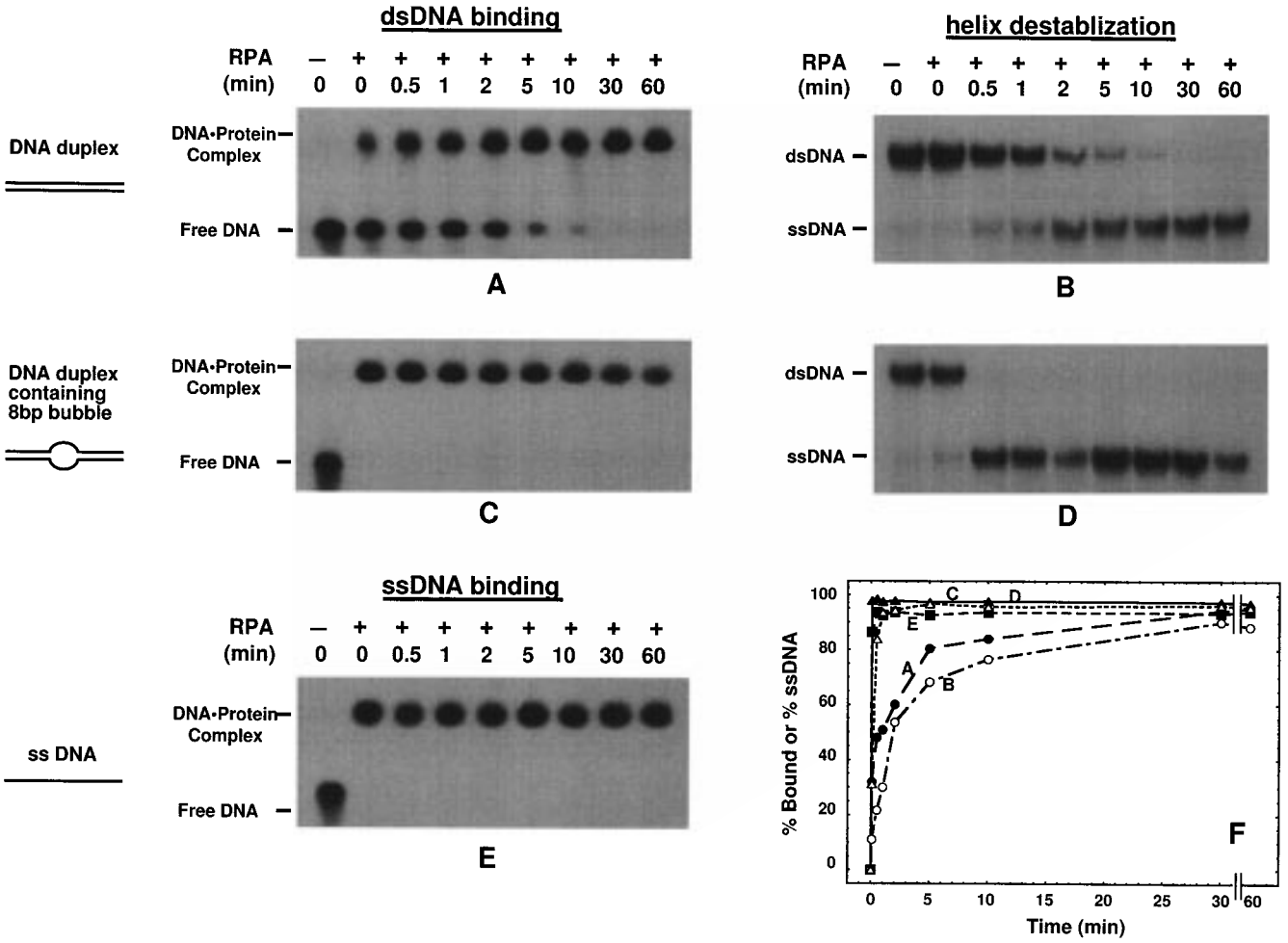

FIGURE 4: Time course of dsDNA-binding (A, C), helix-destabilizing (B, D), and ssDNA-binding (E) activities of RPA. A 3 fmol sample of radiolabeled ssDNA or dsDNA was incubated with 50 fmol of RPA at 25 °C for the indicated period of time. DNA used: (A, B) double-stranded 40 bp fragment; (C, D) double-stranded 40 bp "bubble" fragment; (E) single-stranded 40 nt SV40-top oligonucleotide. Reactions were carried out as described under Materials and Methods. Time courses were quantitated using a Packard Instant Imager, and the percentage of bound DNA or ssDNA is plotted in (F). Data from dsDNA binding (A, long dashes), helix destabilizing (B, dots and dashes), dsDNA binding−bubble substrate (C, solid line), helix destabilizing−bubble substrate (D, short dashes), and ssDNA binding (E, medium dashes).

this hypothesis, we determined the form of DNA present in RPA complexes from a dsDNA binding reaction. RPA−DNA complexes were isolated from an agarose gel (like those shown in Figure 2A). After dislocating the protein−DNA complex with SDS, the DNA was reanalyzed on a polyacrylamide gel. Although the DNA added to the original binding reaction was >95% dsDNA, only ssDNA was found in the RPA complex (data not shown). We conclude that stable binding of RPA to "dsDNA" involves RPA binding to a single-stranded region of the DNA.

RPA can bind to dsDNA containing an internal region of ssDNA or "bubble" (*28*, *59*, *60*). Therefore, a modified form of the 40 bp dsDNA fragment was generated in which 8 nt in the center of the sequence were noncomplementary. This created a dsDNA substrate containing an 8 nt single-stranded "bubble" at its center. When RPA binding to this substrate was examined, both binding and helix destabilization occurred very rapidly (Figure 4C,D); both reactions were complete in <30 s. [The bubble−substrate was observed to be bound even in the 0 min time point (Figure 4C). This observation is probably a consequence of the time necessary to apply reactions to the gel and begin separation in GMSA.] These results suggest that RPA binding to dsDNA requires at least two steps. Initially there is a slow step that involves formation/generation of a small single-stranded region on the DNA. This acts as a "nucleation" point for RPA binding, and then there is a rapid second step in which RPA efficiently destabilizes the helix and forms a stable complex with the resulting larger region of ssDNA. It is not clear whether RPA is directly involved in the initial slow step or whether the initial bubble formation is solely the result of transient ssDNA character caused by thermal fluctuations in the DNA.

Direct Interactions between the Zinc-Finger Motif and DNA. We have shown that mutation of the conserved putative Cys-4 zinc finger present in RPA70 decreases ssDNA-binding (*12*) and dsDNA-binding/helix-destabilizing activity (see above). These data suggest that this zinc-finger motif is directly involved in interactions with DNA. To directly examine this possibility, we incubated different forms of RPA with a radiolabeled 12 nt oligonucleotide and then UV-cross-linked the DNA to the RPA. The high-affinity DNA-binding domain of RPA interacts with ~8 nt (*61*); therefore, the short length of this oligonucleotide should preclude cross-linking with regions of RPA distant from the interaction site. Wild-type RPA was used as a positive control for these assays. As expected with the wild-type complex, the 12 nt oligo-

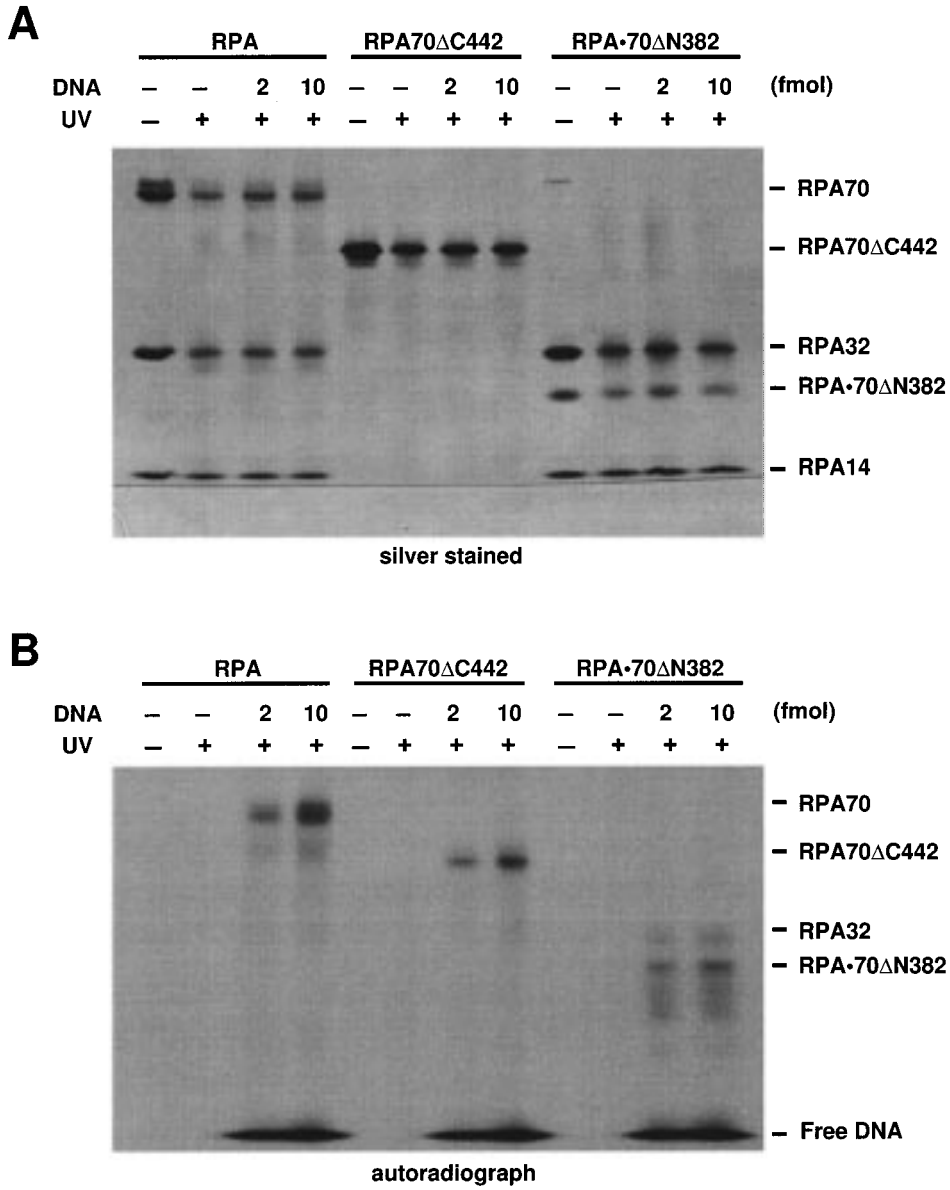

FIGURE 5: UV cross-linking to detect RPA−ssDNA interactions. A 1.5 μg aliquot of RPA, RPA70Δ442, or RPA·70ΔN382 was incubated with the indicated amount of radiolabeled oligonucleotide 12 nt in length (5′-GAGTATTATGAG-3′) in 10 μL of HI buffer containing 30 mM KCl at 25 °C for 20 min. The samples were spotted onto Parafilm and irradiated with UV light at 254 nm for 10 min using UV Stratalinker 2400. The samples were collected and separated on an 8−14% SDS−polyacrylamide gel and visualized (A) by staining with silver nitrate. The presence of RPA−DNA complexes was detected (B) by autoradiography. The positions of RPA subunits and mutants are indicated.

nucleotide was cross-linked to the full-length 70-kDa subunit (Figure 5). RPA70ΔC442 contains the central DNA-binding domain but does not contain the putative zinc-finger motif. This mutant form of RPA was also found to interact and be cross-linked to the 12-mer (Figure 5). To directly examine interactions with the putative zinc finger, we examined RPA·70ΔN382 which is a mutant complex composed of the 32- and 14- kDa subunits and a polypeptide composed of residues 383−616 of RPA70. The complex contains ∼60 residues of the central DNA-binding domain and the putative zinc finger. Initial characterization of this mutant form did not detect high-affinity binding to ssDNA (*34*). However, UV-cross-linking provides a sensitive method to detect medium- and low-affinity interactions. When RPA·70ΔN382 was assayed, the 383−616 polypeptide was cross-linked to the DNA (Figure 5). This indicates that this C-terminal fragment of RPA70 is interacting with ssDNA. To confirm this result, binding of RPA·70ΔN382 was examined in GMSA at very high protein concentrations (∼2 μM). Protein−DNA complexes were observed in these assays; however, it was not possible to saturate binding (data not shown). This suggests that the binding constant of RPA·70ΔN382 must be $<10^5$ M^{-1}. Only one of the seven β strands that make up the DNA-binding OB-fold is present in RPA·70ΔN382 (*61*). This makes it unlikely that binding of RPA·70ΔN382 is due to residual activity of the central DNA-binding domain. Thus, it is most likely that either the zinc-finger motif or some other region of the C-terminal fragment of RPA70 is directly interacting with the DNA. The decrease in ssDNA affinity observed when the zinc-finger motif is mutated (*12*) strongly suggests that the zinc-finger motif is responsible for this interaction.

The 32-kDa subunit of RPA has been shown to interact weakly with ssDNA (*15*, *46*−*49*). In our cross-linking

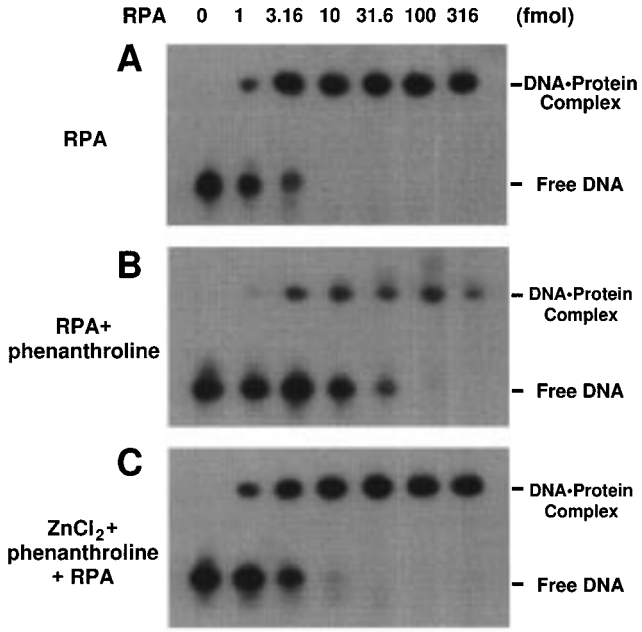

FIGURE 6: Effect of 1,10-phenanthroline on RPA binding. GMSA examining binding of RPA in the presence of 1,10-phenanthroline. Indicated amounts of RPA were incubated with 2 fmol of radiolabeled $(dT)_{30}$ either (A) without pretreatment, (B) after preincubation with 2 mM 1,10-phenanthroline, or (C) after preincubation with a mixture of 2 mM $ZnCl_2$ and 2 mM 1,10-phenanthroline which were mixed together prior to addition of RPA. Preincubations were carried out for 37 °C for 20 min.

studies, we did not detect any cross-linking of the 32-kDa subunit in wild-type RPA (Figure 5). In contrast, cross-linking to RPA32 was observed with RPA•70ΔN382. However, the level of cross-linking was less than that observed with the 383−616 polypeptide of RPA70. These data confirm that the 32-kDa subunit interacts with ssDNA and indicate that the affinity is very weak. In addition, these experiments show that in a complex of heterotrimeric RPA and a short oligonucleotide, RPA32 does not interact with the DNA at a significant level.

To determine whether the putative zinc-finger motif was also interacting with DNA in the heterotrimeric RPA complex, binding studies were carried out in the presence of a strong chelator of divalent cations, 1,10-phenanthroline. RPA binding to oligodeoxythymidine 30 residues in length [$(dT)_{30}$] was examined. Wild-type RPA binds tightly to $(dT)_{30}$ with an apparent binding constant of 4.2×10^{10} M^{-1} (Figure 6A). When the RPA was preincubated with 1,10-phenanthroline, binding was reduced by approximately 1 order of magnitude to 3.8×10^9 M^{-1} (Figure 6B). The effect of 1, 10-phenanthroline was due to chelation of a metal ion because when the 1,10-phenanthroline was preincubated with an equal molar amount of $ZnCl_2$ and then added to an RPA-binding reaction, no change in RPA binding was observed (Figure 6). 1,10-Phenanthroline had no effect on the binding of RPA•70(Zn*) or RPA70ΔC442 (data not shown). Both proteins have an intact central DNA-binding domain but have deleted/mutated zinc-finger motifs. Thus, the effect of phenanthroline is only on the zinc-finger motif. We conclude that the zinc-finger motif is complexed with a metal ion, probably zinc, and that the metal is needed for interactions between this domain and DNA. These interactions occur both in the complete RPA complex and in the absence of the adjacent central DNA-binding domain (see RPA•70ΔN382 above). Furthermore, the decrease in apparent affinity in the presence of 1,10-phenanthroline indicates that the zinc-finger motif contributes 1 order of magnitude to the overall affinity of RPA for ssDNA.

DISCUSSION

RPA has been reported to have multiple types of interactions with DNA. These include: binding ssDNA, dsDNA, and damaged DNA; stimulating T-dependent origin unwinding; and destabilizing dsDNA helices. We have characterized and determined the regions of RPA that are necessary for the dsDNA-binding and helix-destabilizing activities. The central DNA-binding domain of RPA70 was both necessary and sufficient for minimal activity, although other domains are needed for efficient dsDNA binding and helix destabilization. There was a general correlation between these activities and ssDNA-binding activity. Mutant forms of RPA that were defective in ssDNA binding were defective in both other activities. Both dsDNA-binding and helix-destabilizing activities were highly dependent upon the stability of the DNA helix. We also found that dsDNA-binding and helix-destabilizing activities have similar time courses and that both reactions are much slower than ssDNA binding. This slow rate can be dramatically increased by the presence of a ssDNA bubble in the DNA. The simplest explanation for these results is that dsDNA-binding and helix-destabilizing activities are directly related and that both activities are manifestations of RPA's high affinity for ssDNA.

These studies suggest that dsDNA binding is a multistep process consisting of three steps: (1) RPA binds to short ssDNA bubbles in the dsDNA that occur transiently because of localized melting. This step appears to be the rate-limiting step and presumably involves interactions with the central DNA-binding domain of RPA70. (2) RPA unwinds additional sequences to create a 30 nt binding site. This probably involves interactions with additional domains of RPA including the zinc-finger motif. (3) RPA then stably binds to the single-stranded site (Figure 7). The reaction can then either stop or continue with additional RPA molecules binding to completely denature the dsDNA. The amount of ssDNA generated will depend on the DNA sequence and the solution conditions. This mechanism is supported by previous studies showing that RPA can bind to ssDNA fragments as short as 8 nt and to small ssDNA bubbles in dsDNA (*10*, *16*, *28*, *59*, *61*). In one recent study, the structure of the DNA was examined after RPA bound to a dsDNA fragment containing an 8 nt single-stranded bubble (*28*). Under these conditions, the DNA was not completely unwound, but the DNA structure was perturbed on both strands for ∼30 nt surrounding the position of the bubble (*28*).

RPA has been reported to bind to specific dsDNA sequences in yeast (*18*, *19*) and sea urchins (*20*) and to be part of a complex that binds to the human metallothionein promoter in humans (*21*). In yeast, several different families of DNA sequences have been reported to bind RPA with high affinity and high specificity (*18*, *19*). In contrast, we have examined binding of human RPA to multiple dsDNA fragments and have never observed high specificity of binding. However, we have observed differences in binding

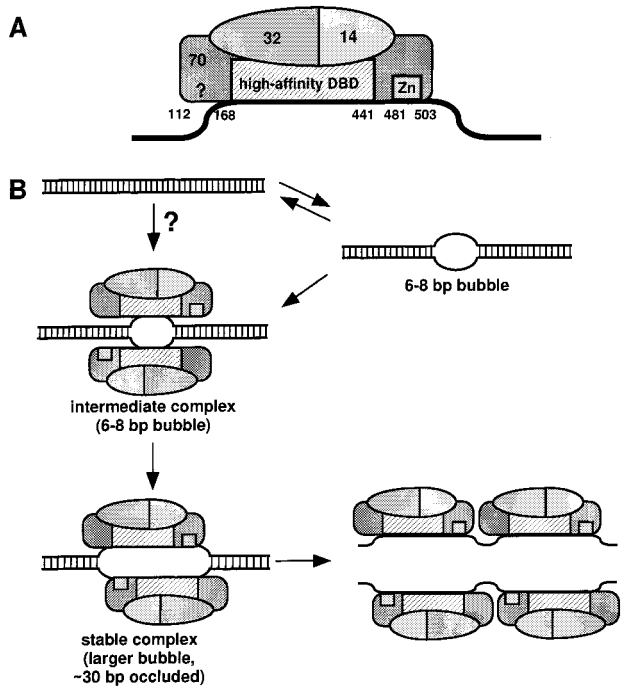

FIGURE 7: Model of RPA binding to dsDNA. (A) Model of RPA interactions with ssDNA. Interactions with central DNA-binding (DBD) and zinc-finger (Zn) domains are shown. Subunits of RPA are indicated by their molecular mass. (B) Model of mechanism of RPA binding to dsDNA. See text for details.

affinity between sequences and found some sequences that RPA bind to weakly. The mechanism of dsDNA binding proposed above provides an explanation of these apparent contradictions. It predicts that RPA will bind with high preference to dsDNA sequences that are easily unwound and that the specificity of RPA binding will be determined by a combination of sequence, solution conditions, and the presence of competitor DNA. This model is supported by the finding that there has not been a dsDNA consensus sequence identified for RPA binding (*4*). This mechanism does not mean that RPA "binding" to dsDNA sequences is not functionally important in the cell. RPA is an abundant protein (*4*), and it is likely to interact with dsDNA in vivo. If RPA interacts with an upstream regulatory sequence, it is likely to affect gene expression regardless of whether the interaction is highly specific or whether the complex produced contains dsDNA or ssDNA.

Prokaryotic ssDNA-binding proteins were initially identified as being helix-destabilizing proteins (*62, 63*). This destabilization (ATP-independent unwinding) of dsDNA is a thermodynamic consequence of binding preferentially to and stabilizing ssDNA. Therefore, it is not surprising that RPA can also destabilize dsDNA. In these studies, we demonstrate that the central DNA-binding domain of RPA70 is responsible for RPA helix-destabilizing activity. Previous analysis of RPA helix-destabilizing ("unwinding") activity indicated that this activity is inhibited by modest ionic strength (high inhibition at 100 mM KCl or 5 mM $MgCl_2$) (*22*−*24*). In agreement with these studies, we found that helix-destabilization and dsDNA-binding activities were reduced by elevated ionic strength and this reduction is correlated with the melting temperature of the DNA. However, both activities were observed at near-physiological ionic strength, indicating that they are likely to occur in the cell.

Deletion of residues between 112 and 168 had a greater effect on helix-destabilizing/dsDNA-binding activity than on ssDNA binding. This suggests that these residues also contribute significantly to this activity. This effect can be explained either by these residues directly interacting with ssDNA or by them stabilizing the structure of the adjacent central DNA-binding domain. Currently, there is no evidence that these residues interact with ssDNA. Furthermore, while deletion of these residues reduced the binding activity of the isolated central DNA-binding domain (*12*), a similar deletion had no effect on ssDNA binding in the context of the entire RPA complex (*11*). This suggests that residues 112−168 play a structural role; however, additional studies are needed to test this hypothesis.

All known homologues of RPA70 possess a highly conserved C_4-type zinc-finger motif in the C-terminal region of the protein. Previous studies on the role of this motif in RPA function have been contradictory with different laboratories reaching opposite conclusions on whether this motif is required for DNA replication and DNA repair (*36, 37, 41, 42*; see also discussion in 12). However, in the accompanying paper we show that this motif is essential for RPA function in SV40 DNA replication and that it is required for optimal ssDNA−DNA-binding activity (*12*). In this paper, we provide a mechanistic basis for these data. The zinc-finger domain directly interacts with ssDNA in the absence of the central DNA-binding domain. Furthermore, incubation with the chelator 1,10-phenanthroline decreases the affinity of RPA but only in forms of RPA that contained an intact zinc-finger motif. This provides the first direct evidence that this highly conserved motif is coordinated with a metal ion. Mutation of the putative zinc-finger motif had a greater effect on reductions in dsDNA-binding and helix-destabilizing activities than on ssDNA binding. This suggests that interactions with the zinc finger are important to helix-destabilizing/dsDNA-binding activity (Figure 7). We hypothesized that interactions with the zinc-finger motif promote the formation of a stable RPA−DNA complex and conversion of dsDNA to ssDNA.

The finding that the zinc-finger domain directly interacts with DNA helps to explain several observations about RPA−DNA interactions. The 32-kDa subunit interacts weakly with ssDNA (*15, 47*−*49*). It was found that a complex containing RPA32, RPA14, and the C-terminal domain of RPA70 had a higher affinity for ssDNA than the RPA32·14 complex alone (*48*). Our data indicate that this higher affinity is due to interaction of the zinc finger with the DNA. It has been hypothesized that binding of RPA32 contributes approximately 1 order of magnitude to the overall binding affinity of RPA (*15, 34*). However, mutation of the zinc-finger motif decreases ssDNA binding to the same extent as deleting RPA32, RPA14, and the C-terminus of RPA70 (e.g., RPA70ΔC442). This indicates either that the zinc-finger motif is responsible for the 1 order of magnitude decrease observed in RPA70ΔC442 or that mutation of the zinc finger disrupts the structure of the RPA complex to prevent RPA32 from contributing to ssDNA binding.

Although RPA has been reported to bind to multiple forms of DNA, these studies demonstrate that ssDNA-binding, dsDNA-binding, and helix-destabilizing activities are all

manifestations of a single activity. In the previous paper, we show that the central DNA-binding domain is necessary for ssDNA binding and that additional regions of RPA are needed for optimal interactions with ssDNA. We have also shown that these regions are responsible for RPA interactions with DNA damage (Lao et al., in preparation). The interactions of RPA with DNA are complex, and a full understanding of RPA function will require elucidation of the molecular basis of these interactions.

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