Recombinant Human Replication Protein A Binds to Polynucleotides with Low Cooperativity†

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ABSTRACT: Replication protein A (RPA) is a multisubunit single-stranded DNA-binding protein that is involved in multiple aspects of cellular DNA metabolism. We have determined quantitative estimates of the binding parameters of human replication protein A (hRPA) from equilibrium binding isotherms. The intrinsic binding constant (K) and cooperativity parameter (ω) were determined from analysis of changes in the intrinsic fluorescence of hRPA that occurred upon binding single-stranded DNA homopolynucleotides. The cooperativity of hRPA binding to both poly(dT) and poly(dA) was found to be low ($\omega = 10-20$) at all NaCl concentrations examined (0.3−2 M). In contrast, the apparent binding affinity ($K\omega$) of RPA decreased significantly with increasing salt concentration, such that log [NaCl]/log $K\omega$ was −2.8 for poly(dT) and −4.8 for poly(dA). We conclude that the salt dependent decrease in binding affinity resulted from changes in the intrinsic binding constant (K). These data suggest that the interaction of hRPA with single-stranded DNA involves significant electrostatic interactions, similar to other single-stranded DNA-binding proteins. The apparent binding affinity ($K\omega$) of RPA was higher for poly(dT) than for poly(dA); extrapolation of our data indicated that the apparent binding affinity at 0.2 M NaCl was 1.6×10^{10} M^{-1} for poly(dT) and 1.1×10^{9} M^{-1} for poly(dA).

Replication protein A (RPA)[1] was originally purified as being essential for SV40 DNA replication (Wobbe *et al.,* 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988). RPA is composed of three subunits of 70, 32, and 14 kDa, that exist as a very stable heterotrimeric complex in solution (Wold & Kelly, 1988; Fairman & Stillman, 1988). Biochemical analysis indicated that RPA has a single-stranded DNA-binding activity and is involved in protein−protein interactions with several other proteins (Wold & Kelly, 1988; Fairman & Stillman, 1988; Kim *et al.,* 1992; Kenny *et al.,* 1990; Dornreiter *et al.,* 1992). RPA has also been shown to participate in both DNA repair and recombination (Coverley *et al.,* 1991, 1992; Heyer *et al.,* 1990; Moore *et al.,* 1991). In addition, RPA may have some involvement in the process of transcription because RPA specifically interacts with acidic domain transcriptional activators such as GAL4 and VP16 (Dornreiter *et al.,* 1992; He *et al.,* 1993; Li & Botchan, 1993). There is also one report that suggests that RPA may be involved in the regulation of the arginase genes in *Saccharomyces cerevisiae* (Luche *et al.,* 1993). Subsequent to the discovery of human RPA (hRPA), homologous proteins have been found in all eukaryotic cells examined. In all cases, RPA homologues are composed of three subunits with conserved primary sequences, have a high affinity for single-stranded DNA, and are important for DNA metabolism (Heyer *et al.,* 1990; Brill & Stillman, 1991; Umbricht *et al.,* 1993; Erdile *et al.,* 1990, 1991; Adachi & Laemmli, 1992; Nakagawa *et al.,* 1991; Fang & Newport, 1993; Brown *et al.,* 1994). In *S. cerevisiae,* the genes for all three subunits have been identified and found to be necessary for viability (Heyer *et al.,* 1990; Brill & Stillman, 1991). This indicates that all three subunits are essential for RPA function. Recently the two smaller subunits of RPA have been shown to form a soluble complex (Henricksen *et al.,* 1994; Stigger *et al.,* 1994). Studies examining the expression and formation of the RPA complex suggest that the smaller two subunits serve a structural role and allow the proper folding of the large subunit and formation of an active RPA complex (Henricksen *et al.,* 1994).

RPA has a high affinity for single-stranded DNA and a much lower affinity for double-stranded DNA and RNA (Wold *et al.,* 1989; Brill & Stillman, 1989; Kim *et al.,* 1992). Thus, RPA fits the classic definition of a single-stranded DNA-binding protein. Human RPA (hRPA) has a binding site of approximately 30 nucleotides, and binding is dependent upon both DNA sequence and DNA length (Kim *et al.,* 1992, 1994; Seroussi & Lavi, 1993). The binding affinity of hRPA varies over 200-fold depending upon the length of the DNA being bound with a maximum affinity for short oligonucleotides on the order of 1×10^{10} M^{-1} (Kim *et al.,* 1994). hRPA has been shown to bind preferentially to polypyrimidine sequences (Kim *et al.,* 1992; Seroussi & Lavi, 1993). Recently, chemical cross-linking experiments by Blackwell and Borowiec have suggested that there may be at least one additional mode of hRPA binding in which hRPA has a smaller binding site (Blackwell & Borowiec, 1994).

Most other known single-stranded DNA-binding proteins bind with relatively high cooperativity (*Escherichia coli* SSB,

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[1] Abbreviations: RPA, replication protein A; hRPA, human replication protein A; rhRPA, recombinant human replication protein A; scRPA, *Saccharomyces cerevisiae* replication protein A; dRPA, *Drosophila melanogaster* replication protein A; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; *E. coli* SSB, *E. coli* single-stranded DNA-binding protein; T4 gp32, T4 gene 32 protein; nt, nucleotide(s).

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$\omega \geq 400$; and T4 gp32, $\omega \sim 2000-3000$; Lohman & Bujalowski, 1990; Karpel, 1990; Ferrari *et al.*, 1994); however, most studies of hRPA and RPA homologues indicate that RPA binds with low cooperativity. Previous studies from our laboratory examining hRPA binding to short oligonucleotides have indicated that hRPA binds with low cooperativity ($\omega \sim 15$) (Kim *et al.*, 1992, 1994). Low levels of cooperativity have also been observed with the RPA homologue from *Drosophila melanogaster* (dRPA)(Mitsis *et al.*, 1993). In contrast, the cooperativity of the RPA homologue from *S. cerevisiae* (scRPA) has been shown to be extremely high ($\omega = 10^4-10^5$) (Alani *et al.*, 1992). In order to define the cooperativity of hRPA binding more completely and to obtain a more detailed understanding of the interactions between hRPA and ssDNA, we have examined hRPA binding to long homopolymers. Quenching of the intrinsic fluorescence of hRPA was monitored to obtain binding isotherms for hRPA at various concentrations of NaCl. We found that hRPA binds with high affinity and low cooperativity to homopolymers. Cooperativity remained constant under a variety of conditions while the intrinsic binding constant decreased significantly as the concentration of NaCl increased.

EXPERIMENTAL PROCEDURES

Polynucleotides. Poly(dT) (2000−4000 nucleotides) and poly(dA) (>1000 nucleotides) were obtained from Midland Co. (Midland, TX). The concentrations of the polynucleotides were determined spectrophotometrically using an extinction coefficient of $\epsilon = 8.1 \times 10^3$ M^{-1} (nucleotide) cm^{-1} at 260 nm for poly(dT) and $\epsilon = 10.0 \times 10^3$ M^{-1} (nucleotide) cm^{-1} at 260 nm for poly(dA) (Kowalczykowski *et al.*, *1981).*

Purification of rhRPA. Human RPA was purified as described (Kim *et al.*, 1994). Recombinant human RPA (rhRPA) was purified from *E. coli* cells expressing all three hRPA genes simultaneously as described previously (Henricksen *et al.*, 1994).

Fluorescence Titrations. Binding reactions and fluorescence titrations were carried out as described previously (Kim *et al.*, 1994) with slight modifications. Binding reactions (1.8 mL) contained the amounts of RPA and DNA indicated and were carried out at 25 °C in buffer F [30 mM HEPES (diluted from 1 M stock at pH 7.8), 5 mM $MgCl_2$, 0.5% inositol, and 1 mM dithiothreitol] with NaCl added to the concentration indicated. To increase the intrinsic fluorescence signal of RPA, a slit width of 2 nm was used. The excitation wavelength was 292 nm, and emission was monitored at 346 nm. These conditions minimize internal filter effects caused by nucleic acids. Under these conditions, we generally observed less than 2% photobleaching during a titration. To reduce experimental variation, cuvettes were freshly siliconized every 10 experiments.

Calculation of Binding Parameters. The theoretical basis for the determination of binding parameters from fluorescence quenching data has been described previously (Kowalczykowski *et al.*, 1986; Lohman & Bujalowski, 1991). The intrinsic fluorescence of hRPA decreases upon binding to ssDNA (Kim *et al.*, 1994). Reverse titrations were carried out in which rhRPA was titrated with ssDNA. Quenching (Q) was calculated as the absolute fluorescence change normalized by the initial RPA fluorescence in the absence of nucleic acids. We assumed for our analysis that quenching was directly proportional to binding [i.e., $Q/Q_{max} = L_b/L_{tot}$; where Q_{max} is maximum quenching, L_b is ligand (rhRPA) bound, and L_{tot} is total ligand]. The titration data were analyzed using the formulation of McGhee and von Hippel (eq 1) (McGhee & von Hippel, 1974, 1976) where ν = the

$$\nu/L = K\cdot(1 - n\nu)\cdot\{[(2\omega - 1)(1 - n\nu) + \nu - R]/$$
$$2(\omega - 1)(1 - n\nu)\}^{n-1}\cdot\{[1 - (n + 1)\nu + R]/2(1 - n\nu)\}^2 \quad (1)$$

"binding density" or moles of bound protein per mole (nucleotide) of nucleic acid lattice (i.e., $\nu = L_b/M_{tot}$ where M_{tot} is the total concentration of lattice), L = the free protein ligand concentration, n = the binding site size (in nucleotides), K = the intrinsic binding constant (in units of M^{-1}), ω = the cooperativity parameter (unitless), and $R = \{[1 - (n + 1)\nu]\cdot 2 + 4\omega\nu(1 - n\nu)\}^{1/2}$. This equation describes the nonspecific binding of a protein to a nucleic acid lattice of infinite length and takes into account both the overlap of potential protein binding sites and the cooperativity of protein binding.

In our experiments, quenching is determined by the level of saturation of the protein rather than the binding density of the nucleic acid lattice (ν); therefore, our data could not be fit directly to eq 1. Instead, theoretical binding isotherms, made up of pairs of values for ν and L, were generated by inserting the independently determined value of $n = 30$ (nucleotides per RPA molecule) (Kim *et al.*, 1992, 1994; see also Figure 2B, below) and test values of K and ω into eq 1. These theoretical values were combined with the known ligand (L_{tot}) and lattice (M_{tot}) concentrations and the observed maximum quenching values (Q_{max}) from individual experiments to generate theoretical quenching curves [$Q = [(L_{tot} - L)Q_{max}]/L_{tot}$] that were functions of the total protein concentration [$M_{tot} = (L_{tot} - L)/\nu$]. (The definitions of Q and M_{tot} were derived directly from the definition of binding density and the assumption that quenching is directly proportional to binding.) Multiple theoretical curves were compared to each experimental data set, and optimal fits were determined by visual inspection.

RESULTS

Initial Characterization of the Binding Properties of rhRPA. Previous studies analyzing the binding properties of hRPA have been limited by the quantities of RPA that could be purified from tissue culture cells. This limitation has been overcome by the recent development of an *E. coli* expression system (Henricksen *et al.*, 1994) which allows the purification of milligram quantities of highly purified (>95% pure) recombinant human RPA (rhRPA). Several lines of evidence suggest that rhRPA has properties similar to the native protein. Hydrodynamic characterization indicated that like hRPA, rhRPA exists as heterotrimer in solution (L. A. Henricksen, personal communication). rhRPA has been shown to bind to ssDNA with the same affinity as native hRPA in gel mobility shift assays and to substitute for hRPA in SV40 DNA replication assays (Henricksen *et al.*, 1994). Our initial characterization of rhRPA was designed to extend these studies and to confirm that the interactions with ssDNA are similar to those of native hRPA. We compared the fluorescence spectra of rhRPA to native hRPA. The intrinsic fluorescence emission spectrum of rhRPA was similar to hRPA (Figure 1). When DNA was

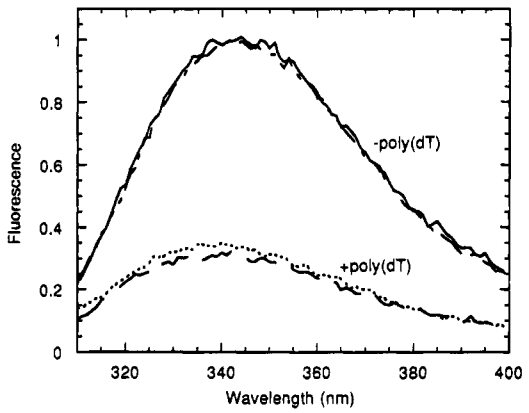

FIGURE 1: Fluorescence emission spectra of hRPA and rhRPA. rhRPA (22.8 nM, solid line) or hRPA (22.5 nM, alternating dots and dashes) was excited at 292 nm in 0.1 M NaCl in buffer F (1.8 mL). Then saturating levels of poly(dT) (3.7 μM nucleotide) were added, and the quenched spectra of rhRPA (broken line) and hRPA (dotted line) were obtained.

added to rhRPA, the fluorescence signal was reduced or quenched (Figure 1). The amount of quenching and the spectra of quenched recombinant protein were very similar to hRPA (Figure 1). These data indicate that the residues responsible for fluorescence are in similar environments in native and recombinant RPA and are consistent with the structure of rhRPA being close to that of hRPA.

In previous studies, we have found that highly purified native hRPA is only partially active for ssDNA binding (Kim *et al.*, 1994). We believe that this partial activity may be due to the chaotropic conditions required to elute hRPA from affinity columns during its purification. rhRPA is purified using similar chromatographic steps as native hRPA. Therefore, in order to use the recombinant protein in quantitative binding studies, it was essential to determine the percent of rhRPA actually capable of binding ssDNA. Two independent methods, stoichiometric fluorescence titrations and equilibrium gel mobility shift assays, were used to determine the activity of rhRPA. In fluorescence assays, a constant amount of rhRPA (102 nM) was titrated with either $(dT)_{20}$ or $(dT)_{30}$ and binding monitored by following quenching of intrinsic rhRPA fluorescence (Figure 2A). We have previously shown that under these conditions, binding of hRPA is stoichiometric (Kim *et al.*, 1994) and that hRPA forms a 1:1 complex with both these oligonucleotides (Kim *et al.*, 1992, 1994). At saturation, the moles of oligonucleotide added are equal to the moles of rhRPA capable of binding ssDNA. With both oligonucleotides, quenching increased linearly with oligonucleotide concentration up to saturation. $(dT)_{20}$ saturated with 53% of the input RPA bound, and $(dT)_{30}$ saturated with 48% bound (Figure 2A). The good agreement between these values is consistent with a 1:1 stoichiometry for both oligonucleotides. These results were confirmed in gel mobility shift assays in which a constant amount of rhRPA was titrated with increasing amounts of radiolabeled oligonucleotide (Kim *et al.*, 1994). Experiments with several oligonucleotides all indicated that this preparation of rhRPA was approximately 50% active (data not shown). Combining the data from all experiments, we conclude that 51% (±3%) of rhRPA was active for DNA binding. This level of activity for rhRPA was similar to preparations of hRPA (Kim *et al.*, 1994). In the studies presented below, all calculations are based upon the concentration of active rhRPA.

The hydrodynamic properties, fluorescence spectra, and DNA-binding properties of rhRPA were all like native hRPA;

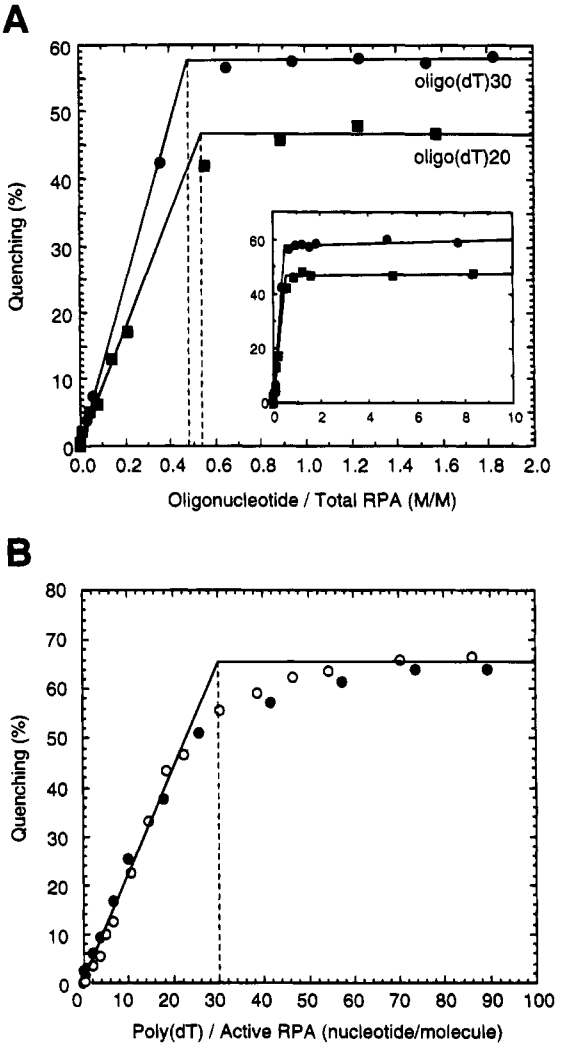

FIGURE 2: Determination of the activity and occluded binding site of rhRPA. (A) Stoichiometric binding reactions (0.1M NaCl in buffer F) in which 102 nM rhRPA (total protein) was titrated with increasing amounts of either $(dT)_{20}$ or $(dT)_{30}$. Data points from the steeply increasing and saturated regions of each titration were independently fit by linear regression, and the resulting lines are shown. Dashed lines indicate the mathematically determined point of intersection of each pair of lines. Inset: An identical plot with an expanded x axis shows data used to define the saturation values of these titrations. (B) Occluded binding site size of rhRPA was determined by stoichiometric reverse titrations in which 51.4 nM active rhRPA (initial concentration) was titrated with poly(dT) (0.1 M NaCl in buffer F). Data from two independent experiments are shown (●, ○); additional data at >100 nucleotides/molecule RPA (not shown) were used to define quenching at saturation. The dashed line indicates the inflection point of the titration.

therefore, we expected rhRPA to possess a binding site size similar to hRPA. To confirm this hypothesis, additional titrations were carried in which poly(dT) was added to a constant amount of rhRPA under stoichiometric binding conditions (0.1 M NaCl). Two such titrations are displayed in Figure 2B. Quenching increased linearly prior to saturation. The round curvature at the inflection point is caused by occlusion of possible binding sites at high levels of protein binding. The binding site size was determined by extrapolating the initial slope to saturation (Figure 2B). The ratio of total poly(dT) to active rhRPA at this intersection, and, thus, the binding site size of rhRPA, was 30 nucleotides. This value was invariant at several concentrations of RPA (50−100 nM active RPA) and NaCl (0.01 and 0.05 M), confirming that the binding of rhRPA was stoichiometric

Table 1: Effect of NaCl on RPA−DNA Interactions

DNA	quenching max (%)	[NaCl] required for 50%reduction in quenching (M)	quenching at 2.5 M NaCl (%)
$(dT)_{15}$	38	0.7	2.5
$(dT)_{30}$	58	2.3	25
poly(dT)	65	2.4	27
$(dA)_{15}$	32	0.45	1
poly(dA)	52	0.75	8.4

under these conditions (data not shown). Moreover, the same site size was obtained when poly(dA) was used instead of poly(dT) (data not shown), indicating that the site size of rhRPA is independent of template used. This suggests that the structure of the complex formed when rhRPA binds to poly(dT) is similar to that formed with poly(dA).

Effect of NaCl on RPA·DNA Interactions. The interactions of rhRPA with several different oligo- and polynucleotides were examined. rhRPA was titrated (at 0.1 M NaCl) with increasing concentrations of DNA and intrinsic quenching monitored. We found that the maximum level of quenching obtained increased with the length of oligonucleotide and that thymidine-containing DNA caused a level of maximum quenching 5−10% higher than deoxyadenosine-containing DNA (Table 1). We then examined the stability of rhRPA·DNA complexes by titration of preformed complexes with NaCl. With all DNAs used, quenching of rhRPA decreased as the NaCl concentration increased. The quenching of intrinsic rhRPA fluorescence caused by $(dT)_{15}$ and $(dA)_{15}$ was completely reversed by ~2 M NaCl (Table 1). In contrast 2.5 M NaCl caused only a partial restoration of the initial fluorescence signal of polynucleotides and long oligonucleotides (Table 1). These changes were not the result of high salt concentration causing a change in the fluorescence properties of rhRPA; the intrinsic fluorescence of rhRPA in the absence of DNA was independent of NaCl concentration up to 2.5 M (data not shown). These data suggest that, like hRPA (Kim *et al.*, 1994), quenching of rhRPA is caused by reversible interactions between rhRPA and DNA and that high concentrations of NaCl disrupt these interactions.

Studies of other single-stranded DNA-binding proteins indicate that the salt stability of protein−ssDNA complexes is usually directly proportional to the affinity for the ssDNA (Lohman & Mascotti, 1992; Overman & Lohman, 1994). Thus, the concentration of NaCl needed to dissociate specific RPA·ssDNA complexes is likely to correlate with the relative affinity of rhRPA for the DNA bound. Similar concentrations of NaCl were required to dissociate both RPA·$(dT)_{30}$ and RPA·poly(dT) complexes (50% dissociation at 2.3 and 2.4 M NaCl, respectively; Table 1), and both complexes were much more resistant to salt than RPA·$(dT)_{15}$ (50% dissociation at 0.7 M NaCl). This suggests that the binding constants of hRPA for $(dT)_{30}$ and poly(dT) are similar and that both are significantly higher than the binding constant for $(dT)_{15}$. These results are consistent with the previous finding that the affinity of hRPA for oligonucleotides increases dramatically with length up to between 30 and 50 nucleotides when the affinity plateaus (Kim *et al.*, 1994). The NaCl concentration needed to restore 50% of the fluorescence signal with RPA·poly(dA) complexes was approximately the same as that required for RPA·$(dT)_{15}$ and much lower than that needed to dissociate RPA·poly(dT) complexes (Table 1), suggesting the affinity of RPA for poly(dA) is lower than for poly(dT).

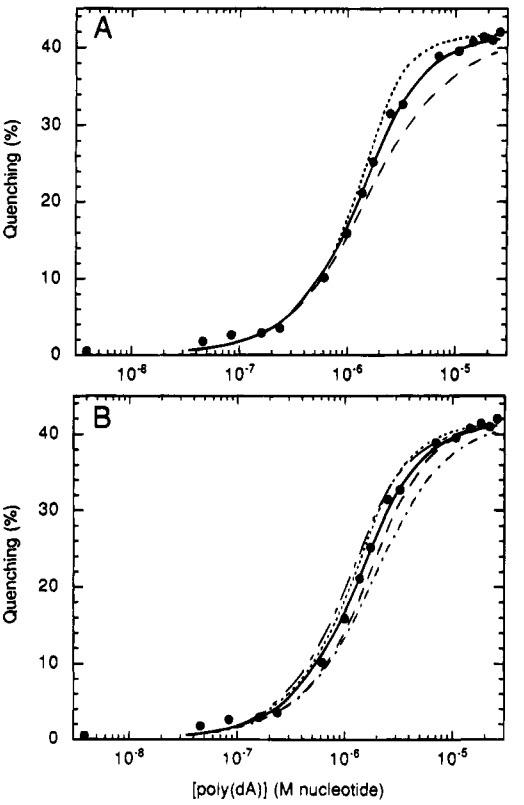

FIGURE 3: Determination of binding parameters from equilibrium binding of rhRPA to poly(dA). rhRPA $(51.4\ nM)_{active}$ was titrated with poly(dA) at 0.4 M NaCl (in buffer F, 25 °C). Quenching was used to monitor the interaction. Black dots are the experimentally determined binding isotherm. Theoretical curves were generated using eq 1. The binding site size was fixed at $n = 30$, and the values of K and ω were varied. 42% quenching was used as maximum quenching for generating theoretical curves. (A) Apparent binding affinity ($K\omega = 3 \times 10^7\ M^{-1}$) was constrained, and both the noncooperative binding constant (K) and cooperativity (ω) were varied. Curves for $K = 2 \times 10^6\ M^{-1}$, $\omega = 15$ (best fit, solid line); $K = 6 \times 10^6\ M^{-1}$, $\omega = 5$ (dotted line); and $K = 6 \times 10^5\ M^{-1}$, $\omega = 50$ (dashed line) are shown. (B) Graph comparing the best theoretical curve ($K = 2 \times 10^6\ M^{-1}$, $\omega = 15$, solid line) to other curves in which either the cooperativity was constrained and the binding constant was varied, $K = 1 \times 10^6\ M^{-1}$, $\omega = 15$ (alternating dots and dashes) and $K = 3 \times 10^6\ M^{-1}$, $\omega = 15$ (dotted line), or the binding constant was constrained and the cooperativity was varied $K = 2 \times 10^6\ M^{-1}$, $\omega = 5$ (dashed line) and $K = 2 \times 10^6\ M^{-1}$, $\omega = 30$ (alternating long and short dashes).

Cooperativity of RPA Binding. In order to determine the binding parameters for rhRPA, it was necessary to examine binding under equilibrium binding conditions. At 0.1 M NaCl, binding of hRPA (at 40−80 nM) to ssDNA is stoichiometric (Kim *et al.*, 1994); however, as shown in the previous section, the binding affinity of rhRPA can be greatly decreased at elevated salt concentrations. Therefore, we carried out equilibrium binding titrations in the presence of high concentrations of NaCl. The resulting equilibrium binding isotherms were analyzed using the cooperative overlap binding model of McGhee and von Hippel (1974). This model accounts for the overlap of potential protein-binding sites on the nucleic acid and for cooperativity in binding. Figure 3 shows equilibrium binding data for rhRPA interacting with poly(dA) at 0.4 M NaCl. The best-fit theoretical curve correlated very well with the data and was generated using an intrinsic binding constant (K) of $2 \times 10^6\ M^{-1}$ and a cooperativity parameter (ω) of 15 (Figure 3, solid line). Other theoretical curves are shown in Figure 3 to illustrate how varying the binding parameters affected the

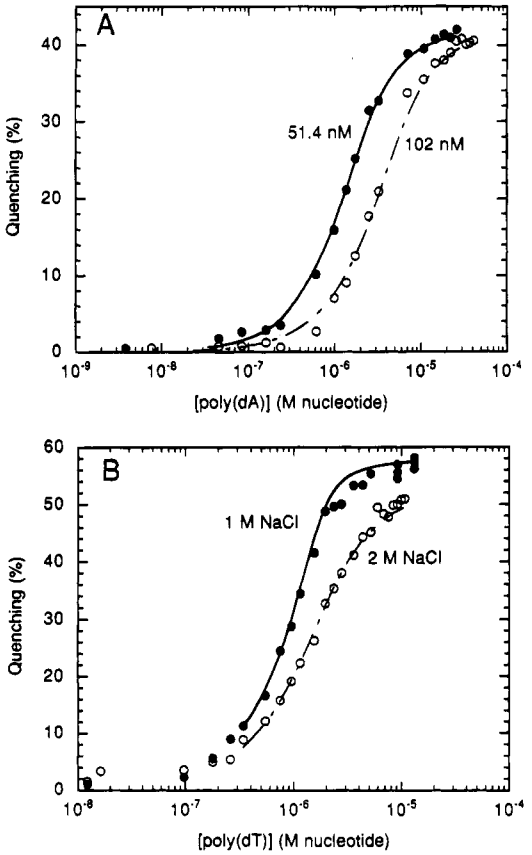

FIGURE 4: Cooperativity is constant at different concentrations of RPA and NaCl. (A) rhRPA [(51.4 nM)$_{active}$ (●) or (102 nM)$_{active}$ (○)] was titrated with poly(dA). Theoretical curves (51.4 nM rhRPA, $K = 1 \times 10^7$ M^{-1}, $\omega = 20$; 102 nM rhRPA, $K = 7 \times 10^5$ M^{-1}, $\omega = 10$) were generated as described in Figure 3. A maximum quenching value of 42% was used to generate theoretical curves for both concentrations of rhRPA (B) rhRPA (51.4 nM)$_{active}$ was titrated with poly(dT) at 1 M (●) and 2 M (○) NaCl (in buffer F, 25 °C). Theoretical curves (1 M NaCl, $K = 1 \times 10^7$ M^{-1}, $\omega = 20$; 2 M NaCl, $K = 1.5 \times 10^6$ M^{-1}, $\omega = 20$) were generated as described in Figure 3. Maximum quenching values of 58% for 1 M NaCl and 53% for 2 M NaCl were used to generate theoretical curves.

correlation with the data. A series of theoretical curves in which either ω or K was varied individually is shown in Figure 3B (dotted and dashed lines). Varying either of the two binding parameters resulted in poorer fit. When the apparent binding affinity ($K\omega$) of RPA was constrained to the best-fit value of 3×10^7 M^{-1} and the cooperativity parameter varied, the curves generated also did not correlate as well with the data (Figure 3A, dotted and dashed lines). We conclude that at 0.4 M NaCl, rhRPA binds to poly(dA) with an affinity of 2×10^6 M^{-1} and a cooperativity of 15.

We also examined binding under other conditions. Figure 4A shows binding isotherms for rhRPA binding to poly(dA) at two different protein concentrations. At both concentrations, the cooperativity of rhRPA binding was low ($\omega = 10-20$). In addition, when binding of rhRPA to poly(dT) was examined, we found that the cooperativity of binding was also low (Figure 4B). All of the conditions used to examine rhRPA interactions with polynucleotides and the binding parameters determined at each condition are summarized in Table 2. These data clearly indicate that using different concentrations of rhRPA, the cooperativity of rhRPA binding to both poly(dA) and poly(dT) was consistently between 10 and 20.

Table 2: RPA Binding Characteristics

lattice	salt (M NaCl)	RPA (nM)[a]	K (M^{-1})	ω	$K\omega$ (M^{-1})	Q_{max} (%)
poly(dT)	0.01	51			S[b]	62
	0.05	51			S	63
	0.1	51			S	65
	1.0	51	1.0×10^7	20	2.0×10^8	58
	1.5	51	3.0×10^6	20	6.0×10^7	55
	2.0	51	1.5×10^6	20	3.0×10^7	53
	2.0	76	9.0×10^5	20	1.8×10^7	53
	2.0	102	8.0×10^5	10	8.0×10^6	53
poly(dA)	0.01	51			S	48
	0.1	51			S	52
	0.2	51			S	51
	0.3	51	1.0×10^7	20	2.0×10^8	43
	0.4	51	2.0×10^6	15	3.0×10^7	42
	0.4	76	5.0×10^5	20	1.0×10^7	42
	0.4	102	7.0×10^5	10	7.0×10^6	42
	0.5	51	6.0×10^5	20	1.2×10^7	40
	0.6	51	5.0×10^5	15	7.5×10^6	39

[a] Initial concentration. [b] S, stoichiometric binding.

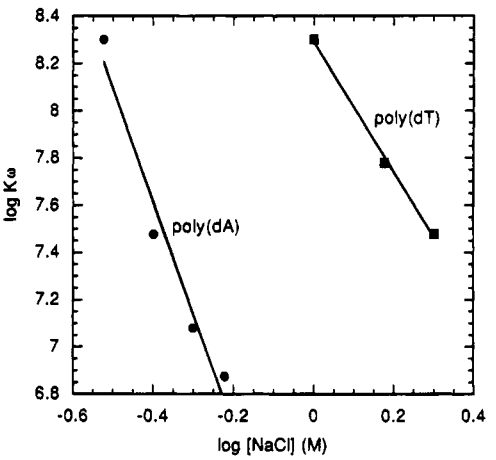

FIGURE 5: Salt dependence of RPA binding to poly(dA) and poly(dT). The binding parameters at 51 nm listed in Table 2 are plotted as log $K\omega$ vs log [NaCl]. Correlation coefficients for fitted lines are $r^2 = 0.964$ for poly(dA) and $r^2 = 0.997$ for poly(dT).

Effect of NaCl Concentration on ω and K. Binding of rhRPA to poly(dT) and poly(dA) at different NaCl concentrations was also examined to assess the effect of salt concentration on the binding parameters of RPA. Figure 4B shows an example of rhRPA·poly(dT) isotherms at 1 and 2 M NaCl. The binding affinity ($K\omega$) decreased 5-fold from $K\omega = 1 \times 10^8$ to 2×10^7 as the salt concentration increased from 1 to 2 M; however, the cooperativity did not change. Similar results were obtained for rhRPA binding to poly(dA) at different NaCl concentrations (Table 2). The cooperativity parameter was determined to be 10−20 at all NaCl concentrations, indicating that ω was independent of NaCl concentration from 0.3 M NaCl up to 2 M NaCl. In contrast, the value of the intrinsic association constant (K) steeply decreased with increasing NaCl concentration. All data obtained at 51 nM rhRPA were combined, and the log of the apparent binding affinity ($K\omega$) was plotted as a function of the log of the concentration of NaCl (Figure 5). For both poly(dT) and poly(dA), a linear relationship between log $K\omega$ and log [NaCl] was obtained. The values of ∂ log $K\omega/\partial$ log [NaCl] were determined to be −4.8 for poly(dA) and −2.8 for poly(dT). Extrapolation of these lines allowed us to estimate the apparent binding affinity for rhRPA at more physiological salt concentrations (Table 3).

Table 3: Salt Dependence of RPA Binding

lattice	$\partial \log K_{app}/\partial \log$ [NaCl]	$K\omega$ 0.1 M NaCl[a]	$K\omega$ 0.2 M NaCl[a]
poly(dT)	−2.8	1.1×10^{11}	1.6×10^{10}
poly(dA)	−4.8	3.0×10^{10}	1.1×10^{9}

[a] Binding constants were determined by extrapolation assuming that the slope of $\partial \log K_{app}/\partial \log$ [NaCl] is linear over the whole salt concentration range in buffer F at 25 °C (see Figure 5).

DISCUSSION

We utilized DNA-induced quenching of rhRPA intrinsic fluorescence to examine the interactions of rhRPA with long homopolynucleotides. We found that RPA·thymidine complexes were more stable at elevated salt concentrations than RPA·deoxyadenine complexes. We also observed that the maximum level of quenching obtained with deoxyadenine was lower than that obtained with thymidine. These differences are consistent with previous studies that demonstrated that RPA binds with ~50-fold higher affinity to pyrimidine-rich sequences than to purine-rich sequences (Kim *et al.*, 1992). We determined the binding parameters of rhRPA by analyzing equilibrium titrations using the model developed by McGhee and von Hippel (1974). We found that the cooperativity of binding to polynucleotides is consistently very low ($\omega = 10-20$) at RPA concentrations between 50 and 100 nM active rhRPA and salt concentrations between 0.3 and 2 M. This value is identical to our previous estimate of cooperativity based on binding to short oligonucleotides at 0.1 M NaCl (Kim *et al.*, 1994).

In contrast to the cooperativity parameter, we found that the apparent association constant ($K\omega$) of rhRPA was very dependent on the concentration of NaCl. Our analysis indicated that the variation of $\partial \log K\omega/\partial \log$ [NaCl] was −4.8 for poly(dA) and −2.8 for poly(dT). The magnitude of these values is comparable to those of T4 gp32 (~−5.9; Newport *et al.*, 1981) and *E. coli* SSB (~−6; Lohman & Bujalowski, 1990). Cooperativity is invariant at different salt concentrations so, like other single-stranded DNA-binding proteins, the salt-dependent variation in the affinity of RPA for ssDNA must be primarily the result of changes in the intrinsic binding constant. Extrapolation of our equilibrium binding data to 0.1 M NaCl predicts an apparent association constant for rhRPA for poly(dT) of 1.1×10^{11} M^{-1} (Table 3) and an intrinsic binding constant ($K\omega/\omega$) for poly(dT) at 0.1 M NaCl of 7.3×10^{9} M^{-1}. This value correlates very well to the intrinsic binding constants determined previously for hRPA binding to $(dT)_{30}$ ($K = 4.6 \times 10^{9}$ M^{-1}) and $(dT)_{50}$ ($K = 15 \times 10^{9}$ M^{-1}) (Kim *et al.*, 1994). The strong dependence of $K\omega$ on NaCl concentration indicates that there are appreciable electrostatic interactions when RPA binds to ssDNA.

RPA homologues from calf thymus and *Drosophila* have binding site sizes of 20−30 nucleotides and bind ssDNA with low cooperativity ($\omega = 10-50$) (Table 4; Atrazhev *et al.*, 1992; Mitsis *et al.*, 1993). In contrast, studies of scRPA in 140−160 mM $MgCl_2$ indicated that scRPA has a much larger binding site ($n = 90-100$) and binds with very high cooperativity ($\omega = 10^4-10^5$) (Alani *et al.*, 1992). Extrapolation of these data indicates that the apparent binding constant of scRPA at 13 mM $MgCl_2$ should be $\sim 1 \times 10^{9}$ M^{-1} (Alani *et al.*, 1992) and, assuming that the cooperativity of scRPA is invariant with salt concentration, that the intrinsic binding constant should be 10^4-10^5 M^{-1}. scRPA and hRPA are highly homologous; therefore, these differences in binding properties probably reflect alternate modes of binding of RPA under different conditions. There is evidence that RPA−RPA interactions can occur in solution and that RPA can form oligomers or aggregates under some conditions (Alani *et al.*, 1992; Mitsis *et al.*, 1993; Kim *et al.*, 1994). These protein−protein interactions could increase the apparent cooperativity of RPA binding under certain conditions. A recent study also suggested that hRPA may have an alternate mode of binding with an 8−10 nucleotide-binding site (Blackwell & Borowiec, 1994). This binding mode occurred under certain salt conditions at high concentrations of hRPA and required chemical cross-linking to be observed (Blackwell & Borowiec, 1994). We have observed no evidence for this mode of binding in these fluorescence studies or previous hydrodynamic studies of hRPA·DNA complexes (Kim *et al.*, 1994). Potentially, this "10-nucleotide" binding mode could only occur under certain solution conditions as has been observed for the alternate modes of binding of *E. coli* SSB (Lohman & Bujalowski, 1990). A second possibility is that the "10-nucleotide" binding mode may be mediated through hRPA−hRPA interactions that are stabilized by chemical cross-linking.

Table 4: Summary of ssDNA Binding Properties of RPA Homologues

source	site size	cooperativity	K (0.2 M^{-1})	$K\omega$ (0.2 M^{-1})	Q_{max}[k]
human[a]	30	10−20[e]	~2×10^{9}[h]	4×10^{10}[j]	65[h]
yeast[b]	90−100	10^4-10^5[f]	ND	ND	65
Drosophila[c]	22	10−300[g]	~5×10^{5}[i]	$(0.5-5) \times 10^{7}$[i]	35[i]
calf thymus[d]	23	ND[l]	ND	ND	ND

[a] This study; Kim *et al.* (1994). [b] Alani *et al.* (1992). [c] Mitsis *et al.* (1993). [d] Atrazhev *et al.* (1992). [e] [NaCl] 0.3−2 M. [f] [$MgCl_2$] 160−180 mM. [g] 0.22 M NaCl. [h] Poly(dT) as template, $K = K\omega/\omega$. [i] M13 ssDNA used as template. [j] By extrapolation using poly(dT). [k] Fluorescence quenching maximum. [l] ND, not determined.

Most single-stranded DNA-binding proteins bind with relatively high cooperativity [e.g., T4 gp32 (ω = ~2000; Kowalczykowski *et al.*, 1981), fd gene 5 protein ($\omega = 400-800$; Pörschke & Rauh, 1983), Herpes ICP8 (ω = ~1000; Williams & Chase, 1990) and *E. coli* SSB (ω = ~400 for $(SSB)_{65}$ and $\omega = \sim 10^5$ for $(SSB)_{35}$; Bujalowski & Lohman, 1987; Lohman & Ferrari, 1994)]. However, RPA is not unique in binding to ssDNA with low cooperativity. Adenovirus DNA-binding protein ($\omega = 25$) and retroviral ssDNA-binding protein ($\omega = 1$) both also bind with low cooperativity (Kuil *et al.*, 1989; Karpel *et al.*, 1987). Cooperativity has two primary effects on the binding of a protein to ssDNA. First, cooperativity increases the apparent binding constant so that the protein will have a higher affinity for long stretches of ssDNA. Second, cooperativity increases the chances of molecules binding adjacent to each other, increasing the clustering of the protein on DNA. Both of these properties promote efficient coating of ssDNA which is believed to be one of the primary functions of single-stranded DNA-binding proteins in the cell. In the case of hRPA, we believe that high cooperativity is not needed for this function. The number of molecules of RPA in a human cell has been estimated to be between $(4-5) \times 10^4$ (Wold & Kelly, 1988; Kenny *et al.*, 1990) and 2.4×10^5 (Seroussi & Lavi, 1993). This translates to a cellular RPA concentration (at least 0.1−1 μM) sufficient to produce stoichiometric binding conditions in the cell, and, thus, any ssDNA present

would be rapidly saturated by RPA independent of the level of cooperativity of binding.

RPA interacts specifically with several other proteins including SV40 T antigen and DNA polymerase α (Dornreiter *et al.*, 1992). These interactions seem to be essential for the initiation of SV40 DNA replication (Collins & Kelly, 1991; Murakami *et al.*, 1992; Melendy & Stillman, 1993) and may be important for coordinating DNA metabolism with other cellular processes (Li & Botchan, 1993; He *et al.*, 1993; Dutta *et al.*, 1993). Since cooperative binding normally arises through homologous protein−protein interactions, cooperativity in RPA binding would probably require significant RPA−RPA interactions. Such interactions could compete with heterologous RPA−protein interactions. Thus, high levels of cooperativity could inhibit heterologous interactions necessary for normal RPA function. It should be noted, however, that T4 gp32 is able to participate in specific protein−protein interactions in addition to having high cooperative binding to ssDNA (Karpel, 1990).

These studies and previous research from our laboratory have provided initial estimates of the binding parameters of hRPA (Kim *et al.*, 1992, 1994). A complete understanding of the function of RPA in the cell during DNA replication, repair, and recombination will require additional characterization of the interactions of RPA with DNA and with other proteins. In order to understand the role of cooperativity in RPA function, it will be essential to determine the nature and prevalence of alternate modes of binding of RPA to ssDNA and the relative importance of homologous RPA−RPA versus heterologous RPA−protein interactions.

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