Independent and Coordinated Functions of Replication Protein A Tandem High Affinity Single-stranded DNA Binding Domains

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The initial high affinity binding of single-stranded DNA (ssDNA) by replication protein A (RPA) is involved in the tandem domains in the central region of the RPA70 subunit (RPA70AB). However, it was not clear whether the two domains, RPA70A and RPA70B, bind DNA simultaneously or sequentially. Here, using primarily heteronuclear NMR complemented by fluorescence spectroscopy, we have analyzed the binding characteristics of the individual RPA70A and RPA70B domains and compared them with the intact RPA70AB. NMR chemical shift comparisons confirmed that RPA70A and RPA70B tumble independently in solution in the absence of ssDNA. NMR chemical shift perturbations showed that all ssDNA oligomers bind to the same sites as observed in the x-ray crystal structure of RPA70AB complexed to d(C)12. Titrations using a variety of 5′-mer ssDNA oligomers showed that RPA70A has a 5–10-fold higher affinity for ssDNA than RPA70B. Detailed analysis of ssDNA binding to RPA70A revealed that all DNA sequences interact in a similar mode. Fluorescence binding measurements with a variety of 8–10-mer DNA sequences showed that RPA70AB interacts with DNA with ~100-fold higher affinity than the isolated domains. Calculation of the theoretical “linkage effect” from the structure of RPA70AB suggests that the high overall affinity for ssDNA is a byproduct of the covalent attachment of the two domains via a short flexible tether, which increases the effective local concentration. Taken together, our data are consistent with a sequential model of DNA binding by RPA according to which RPA70A binds the majority of DNA first and subsequent loading of RPA70B domain is facilitated by the linkage effect.

The fundamental genetic processes of DNA replication, recombination, and repair are carried out by large multiprotein assemblies. Single-stranded DNA-binding proteins are required to organize and protect exposed ssDNA in all of these pathways. The single-stranded DNA-binding protein function in eukaryotes is carried out by the heterotrimeric replication protein A (RPA). The three subunits (RPA70, RPA32, RPA14), named according to their respective molecular weights, are highly conserved, and all are required for function (1, 2). Three-dimensional structures determined by NMR and x-ray crystallography have been obtained for all RPA domains, but the overall quaternary structure of the trimer is not yet known. A schematic representation of the domain organization of RPA is presented in Fig. 1. The N- and C-terminal regions of RPA70 (RPA701–110, RPA70N; RPA70 436–616, RPA70C), the central region of RPA70 (RPA70181–422, RPA70AB), the central domain of RPA32 (RPA3245–171, RPA32D), and RPA14 all adopt an oligonucleotide/oligosaccharide binding fold, a structure common to other known single-stranded DNA-binding proteins (3–7). The C-terminal region of RPA32 (RPA32205–270, RPA32C) contains a winged helix-loop-helix domain (8). Binding of ssDNA is carried out by RPA70A (RPA70181–291), RPA70B (RPA70208–422), RPA70C, and RPA32D. RPA70N and RPA32D do not contribute to DNA binding but are involved in protein-protein interactions with other DNA replication, recombination, and repair proteins (1, 9).

RPA binds to ssDNA with an association constant of ~1010 M−1 (10). There is a measurable preference for polypurine over polypyrimidine over polypurine sequences, as indicated by a 50–100-fold stronger binding constant (11). The domain arrangement is dictated by the polarity of the ssDNA, with domains A through D binding from the 5′- to the 3′-end of a given sequence (12–14). Each heterotrimer binds in three stages, with occlusion lengths of 8–10, then 13–14, and finally ~30 nucleotides (15, 16). The initial stage of binding has been attributed to the tandem high affinity domains RPA70AB (7). However, it is not clear whether the individual A and B domains bind DNA simultaneously or sequentially.

The x-ray crystal structure of RPA70AB in complex with d(C)12 has provided some fundamental insights into the binding of ssDNA (4). The most striking feature was the observation of base-specific contacts between the protein and DNA. These include a network of hydrogen bonds and base stacking interactions of conserved aromatic residues in L12 and L45 loops in both domains. These base-specific contacts are seemingly inconsistent with the need for RPA to bind ssDNA regardless of sequence information. A hypothesis has been proposed to explain sequence non-specific binding of ssDNA by RPA70AB, which invokes dynamic remodeling of the binding sites in ac-

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‡ The abbreviations used are: ssDNA, single-stranded DNA; RPA, human replication protein A; HSQC, heteronuclear single quantum coherence.

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The backbone amide proton and nitrogen chemical shifts of intact and unlabeled or [13C6]glucose was used as the sole nitrogen and carbon phosphorylation. Zn and side chain assignments of RPA70A and RPA70B (20). The 1H-15N ard set of triple resonance experiments was acquired for the backbone of the individual domains. This strategy was based on the observation similar manner except that M9 minimal medium containing 15NH4Cl without further purification. All other chemicals were of molecular geneity of all samples were tested using SDS-PAGE and matrix-as-

all NMR experiments was 20 mM Tris-d11 HCl containing 50 mM KCl, 10 Bruker spectrometers operating at 600 or 800 MHz. The buffer used for

gel filtration column. The final yield is 15

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host BL21 (DE3) pLys(S), and

were expressed in the Escherichia coli host BL21 (DE3) pLyS(S), and RPA70AB was expressed in BONETTA BL21 (DE1) cells (Novagen, Madison, WI). Unlabeled proteins were prepared using LB medium containing ampicillin (or carbenicillin in the case of RPA70AB) at 37 °C. Uniform 15N- and 13C, 15N-labeled protein was produced in a similar manner except that M9 minimal medium containing 15NH4Cl and unlabeled or [15C]glucose was used as the sole nitrogen and carbon source. Proteins were purified by nickel-nitrilotriacetic acid affinity chromatography. The His tag was cleaved off for RPA70A and RPA70B but retained in the case of RPA70AB for stability reasons. Further purification was achieved using Superdex-75 (Amersham Biosciences) gel filtration column. The final yield is 15–20 mg/liter for culture for RPA70A and RPA70B and ~8 mg/liter for RPA70AB. The yield of the protein under labeling conditions dropped by ~50%. Purity and homogeneity of all samples were tested using SDS-PAGE and matrix-as-
sister desorption ionization mass spectroscopy.

High performance liquid chromatography-purified ssDNA samples were purchased from Midland Certified Co., Midland, TX and used without further purification. All other chemicals were of molecular biology grade.

NMR Spectroscopy—All NMR experiments were performed on Bruker spectrometers operating at 600 or 800 MHz. The buffer used for all NMR experiments was 20 mM Tris-d1, HCl containing 50 mM KCl, 10 mM MgCl2, 2 mM dithiothreitol, and 0.01% NaN3 at pH 7.2. All NMR experiments were recorded at 25 °C. Two-dimensional, gradient-en-
hanced 1H-15N HSQC spectra were acquired on uniformly 15N-labeled samples of RPA70A, RPA70B, and RPA70AB in 90% H2O/10%D2O (19). The backbone amide proton and nitrogen chemical shifts of intact RPA70AB were assigned by comparison with the 1H-15N HSQC spectra of the individual domains. This strategy was based on the observation that the two domains are structurally independent (see below). A standard set of triple resonance experiments was acquired for the backbone and side chain assignments of RPA70A and RPA70B (20). The 1H-15N HSQC spectrum of RPA70AB with the backbone assignments is presented as supplementary figure S1. Complete backbone and side chain assignments for RPA70A, RPA70B, and RPA70AB will be reported elsewhere (21). All NMR spectra were processed and analyzed using Felix 2000 (Accelrys Inc., San Diego, CA) or

ssDNA Titrations by NMR—The following titrations were carried out: (i) RPA70A with dCTTCA, dC(T)A, and d(T)3; (ii) RPA70B with dCTTCA, dC(T)A, and d(T)3; (iii) RPA70B with dCTTCA, dC(T)A, and d(T)3; (iv) RPA70B with dCTTCA, dC(T)A, and d(T)3.

The protein concentration used was between 0.15 and 0.35 mM. Aliquots of ssDNA were added to the protein and equilibri-

ated for 5 min before acquiring 1H-15N HSQC spectra. The pH of the NMR samples was monitored during the titration, and there was no significant change in the pH at the end of each titration (within ± 0.2 units).

Changes in average amide chemical shifts (Δδavg) were calculated using

where ΔNN and ΔH are the amide nitrogen and amide proton chemical shift difference between the free and the bound states of the protein (22). NMR chemical shift changes were fit using the same procedure that was followed to fit the fluorescence binding curves.

Fluorescence Spectroscopy—RPA70A and RPA70B each contain two tryptophans. Importantly, one tryptophan in each domain is exposed to solvent and directly involved in the DNA binding (Trp-212 and Trp-361 in A and B, respectively), whereas the others, Trp-197 and Trp-414, are buried and far from the binding site. The location of residues in the binding site suggested that analysis of the quenching of tryptophan fluorescence could be used to monitor the binding of ssDNA within each domain, and experiments reported here confirm the validity of this approach.

Steady-state fluorescence experiments were done using a SPEX FLUOROMAX spectrofluorometer. Emission spectra were recorded by scanning between 305 and 400 nm with the excitation wavelength for tryptophan set at 285 nm. Excitation and emission slit widths were set to 4 nm.

Fluorescence measurements were carried out at ambient temperatures in a 160-μl-volume quartz cuvette using a protein concentration of 10–20 μM. The buffer used for fluorescence experiments was 20 mM HEPES containing 50 mM KCl and 10 mM MgCl2 at pH 7.2. Titrations were performed by the addition of aliquots of ssDNA from a concent-

ated stock solution. The overall volume change during the titration was ≤5% of the total reaction volume and was corrected in the final analysis. At each titration point, the sample was incubated for 2 min before recording the spectra.

The changes in the fluorescence intensity were measured at the maximum intensity (Fmax) of 337 nm for RPA70AB, 333 nm for RPA70A, and 343 nm for RPA70B, respectively. Fluorescence intensities were fit using KaleidaGraph 3.5 (Synergy Software) to a simple bimolecular binding model through non-linear regression analysis using Equation 2,

where P, is the total concentration of protein, D, is the total concentration of DNA, and Kd is the dissociation constant of the complex. In the final equation, both the starting and the end points of the titration were normalized to 0 and 1, hence Fmax becomes 0 and F400 max is 1. The binding constants reported in this study were determined using a protein concentration within the measurable range of Kd values as suggested by simulated binding curves. NMR experiments could be used to determine Kd values in the range 2–40 μM, and fluorescence could be used to determine Kd values in the 50 mM to 5 μM range.

Local Concentration (Linkage) Effect—The local concentration effect was calculated based on modeling the system as a wormlike chain following the procedure published by Zhou (23). The linker length is 27 residues for RPA70AB. The end-to-end distance measured in the crystal structure (33.7 Å) was used for this calculation.

RESULTS AND DISCUSSION

A and B Are Structurally Independent Domains—The x-ray crystal structure of RPA70AB in the presence of d(C)8 revealed that although the two domains are aligned, they are actually very few contacts between them (4). In the absence of ssDNA, the two domains are no longer aligned, and no interdomain contacts are observed (5). In fact, one of the molecules in the unit cell has only partial electron density for the linker between the two domains. To properly interpret the DNA binding properties of RPA70AB, it is important to establish whether this
The RPA70A Domain Binds ssDNA with Higher Affinity Than the RPA70B Domain—Both NMR and fluorescence spectroscopy were used to monitor the relative binding of ssDNA by the A and B domains. A five-nucleotide oligomer, d(CTTCA), which covers a single binding site, was used for these experiments. Upon titration of the DNA into a solution of $^{15}$N-enriched protein, a subset of cross-peaks in the $^1$H-$^{15}$N HSQC NMR spectrum is perturbed, indicating binding in a discrete binding site on the protein. The availability of sequence-specific resonance assignments allowed the ssDNA binding site to be determined in each case. As expected, the results obtained were fully consistent with the x-ray crystal structure of the complex of RPA70AB with d(C)_8, in which residues in the L12 and L45 loops (Trp-212–Glu-218, Lys-263–Glu-275) and part of the β region (Arg-234–Phe-238) are in direct contact with DNA in each domain and with RPA70A and RPA70B oriented toward the 5’- and 3’-end of DNA, respectively.

The NMR chemical shifts of RPA70A, RPA70B, and RPA70AB are perturbed as d(CTTCA) is titrated into the solution. The observation of fast exchange for all perturbed residues greatly facilitated the assignment of the NMR chemical shifts in the DNA-bound state by starting with the complete assignments for the free protein and simply tracking the small shifts in resonances at each step of the titration. A binding curve for representative residues shows significant chemical shift changes upon the addition of increasing amounts of d(CTTCA) (Fig. 3). As seen in Fig. 3A, binding of d(CTTCA) to RPA70A saturated around a 1:1 molar ratio of DNA to protein, indicating 1:1 stoichiometry. An upper limit on $K_d$ of 2 μM was estimated using the 5 residues showing the largest total chemical shift change (Glu-218, Ala-237, Phe-238, Glu-268, Ala-271). This NMR measurement is fully consistent with the value of 1.7 ± 0.8 μM determined by fluorescence spectroscopy (data not shown). Interestingly, the titrations for RPA70B show that the binding is weaker as reflected in the initial slope of the binding curve and saturation at a higher DNA:protein ratio (Fig. 3B). An average $K_d$ of 16.8 ± 3.2 μM was derived from the 5 residues showing the largest total chemical shift change (Ser-336, Arg-382, Phe-386, Arg-389, Leu-391, Ser-395). These data reveal a small but significant (8-fold) difference in the binding affinities of isolated RPA70A and RPA70B for d(CTTCA).

A corresponding NMR titration of d(CTTCA) into a solution of $^{15}$N-enriched RPA70AB was also carried out. A good correlation is found between the peaks appearing in the NMR spectrum of the DNA-saturated protein and the corresponding peaks in the spectra of the isolated domains saturated with DNA (Fig. 2), consistent with the functional independence of the two domains. The chemical shift changes from a representative set of residues from both the A and the B domains are shown in Fig. 3C. The binding curves represent the summation of two simultaneous independent binding events, and in particular, enable a direct observation of the relative affinities of the two domains. The binding curves for the residues from the A domain appear monophasic. In contrast, residues from the B domain clearly show two phases of binding. These data reflect ssDNA loading preferentially into the A domain, which is saturated before the B domain is even half-loaded. This provides a clear and direct evidence of the higher intrinsic binding affinity of the A domain even in the context of the tandem domain, RPA70AB.

Common Binding Mode—To establish that the observed difference in affinity of RPA70A and RPA70B is a general phenomenon and not an artifact specific to d(CTTCA), the experiments were repeated with d(C)_n and d(T)_n. As anticipated, the
results obtained for this oligomer were the same within the limits of experimental error (data not shown).

NMR chemical shift analysis was also carried out to monitor the effect on the structure of RPA70A upon binding to various 5-mer sequences. The NMR spectra of RPA70A bound to d(C)₅ or d(T)₅ shows a striking resemblance to that observed for a mixed sequence such as d(CTTCA) (Fig. 4). A similar result was observed when d(C)₁₀ or d(CTTCACTTCA) was titrated with RPA70AB (data not shown). This strongly suggests that the protein adopts a very similar structure upon binding to different ssDNA molecules. However, as shown in Fig. 5, there are also some specific differences in the magnitude of the chemical shift change upon binding to various sequences. For example, the peak for Ala-237 in RPA70A changes significantly upon binding to d(C)₅ and d(CTTCA) but only very little when bound to d(T)₅, whereas the peak for Ala-271 shows significant changes upon binding to all the three sequences. These localized changes are due to the adaptation of side chains to different DNA sequences, as proposed previously (17), and are also presumably responsible for the higher affinity of RPA70A for ssDNA.

Coordinated Binding of ssDNA in Intact RPA70AB—DNA binding by the A and B domains involves integral contact with 3 nucleotides each. Consequently, the studies of binding of the d(CTTCA) oligomer to RPA70AB described above provide information only on the relative intrinsic affinities of the two domains and not on the overall binding properties of RPA70AB. Since the A and B domains of RPA70AB are linked to each other by a tether, their binding of DNA is perforce coordinated. The well defined relative orientation of the two domains in the x-ray crystal structure of the DNA complex clearly points to coordinated binding, although the dearth of contacts between the two domains suggests little if any cooperativity between the two domains. To confirm that this specific arrangement of the domains is not simply a byproduct of the rather short length (8 nucleotides) of the DNA oligomer required for crystallization, the binding affinities for d(C)₅ and d(CTTCA) were measured by intrinsic tryptophan fluorescence and the structure of the corresponding complexes compared by heteronuclear solution NMR.

Attempts at determining the DNA binding affinities of RPA70AB by NMR were stymied by exchange effects on the
of 10nM determined by electrophoretic mobility shift assay.

These characteristic backbone and side chain 1H, 15N, and 13C NMR chemical resonances that are not observable in the middle of the titration. There is, however, a clear quenching of intrinsic RPA70AB tryptophan fluorescence accompanied by a small but distinct blue shift upon binding DNA (Fig. 6, A and B). Fitting of the curves to a simple binding equation results in dissociation constants of 52 ± 16 nM, 44 ± 12 nM, and 114 ± 32 nM for the d(C)₈, d(C)₁₀, and d(CTTCACTTCA), respectively. These are >100-fold stronger than the values of 2–10 μM for the DNA 5-mers. The Kₐ value measured for d(C)₈ is similar to the value of 10 nM determined by electrophoretic mobility shift assay (18). No uncertainties were provided for the latter experiments, but if the differences in these values are real, they are presumably due to the differences in the experimental conditions and the type of oligonucleotides used in these studies (in the latter case, 5'-phosphorylated d(C)₈ was used, whereas in the present study, the oligonucleotides were not phosphorylated).

The similarity of the Kₐ values of d(C)₈ and d(C)₁₀ confirms that the isolated tandem RPA70AB domains specifically interact with a segment of 8 nucleotides, regardless of the length of the oligonucleotide presented. Detailed comparison of characteristic backbone and side chain ¹H, ¹⁵N, and ¹³C NMR chemical shifts shows that there are no differences in the corresponding structures of RPA70AB. In addition to the dC homo-oligomers, corresponding experiments were carried out on d(CTTCACTTCA), and essentially identical binding affinities (Fig. 6) and chemical shift changes in the spectrum were observed.

Local Concentration, the Linkage Effect—What factors lead to the observed ~100-fold difference in the affinity of ssDNA depending on whether or not the two domains of RPA70AB are linked? Allosteric coupling in systems with multiple binding sites is well known; is the binding of ssDNA to RPA70AB cooperative? To evaluate cooperativity in a system such as RPA70AB, it is essential to consider the intrinsic contribution to binding affinity due to the covalent linkage of the two domains. In essence, attachment leads to an increased local concentration of the two domains, relative to the free diffusion of independent molecules.

To determine this “linkage effect,” we have used the formalism of Zhou (23), which is based on polymer theory and assumes that the flexible linker can be modeled as a worm-like chain with minimal interference between the linker and the domains. A schematic diagram representing this model and the thermodynamic equilibrium involved for the RPA70AB system is presented in Fig. 7. Using this formalism, the local concentration ρ(d₀) for RPA70AB was calculated to be 2.3 mM. This value agrees well with the local concentration that can be derived from comparison of the experimentally determined dissociation constants for the individual domains (RPA70A and RPA70B) in complex with d(CTTCA) (i.e. in the absence of linker, Kₐ and KₐB) and the intact RPA70AB domain in complex with d(CTTCACCTTCA) (KₐB). This conclusion is consistent with our structural comparisons of RPA70AB by NMR, which show that the two domains are structurally independent both in the absence and in the presence of DNA. These results imply that although they function in a coordinated fashion, there is no significant cooperativity when the two domains interact with DNA.

Concluding Remarks—The experiments reported in this study provide insights into the coordinated ssDNA binding activities of the RPA70A and RPA70B domains, as well as the similarity in the mode of binding of different DNA sequences. To facilitate the comparisons of complexes with different sequences and with the crystal structure of the d(C)₈ complex, the ssDNA oligomers used in this study were all pyrimidine-rich. A corresponding analysis of purine-rich and mixed purine-pyrimidine sequences is clearly necessary to obtain a complete picture of the RPA70AB DNA binding properties and refine our hypotheses. These and additional crystallographic studies should also shed light on the molecular basis for the higher apparent affinity of RPA for polypyrimidine versus purpurine DNA.

RPA uses four oligonucleotide/oligosaccharide binding fold domains to bind ssDNA. The tandem domain, RPA70AB, contributes over 80% of the overall affinity of RPA to ssDNA. The A domain has higher intrinsic affinity for ssDNA than the others, but the short linker to the B domain ensures that the binding activities of the tandem domains are coordinated, resulting in high overall affinity. The RPA70B and RPA70C domains are also connected by a relatively short linker (resi-
dyes —422–436), and it will be important to determine whether this linker plays a similar role.

Our data regarding the difference in binding affinity of domains A and B correlates with mutational analysis results. In vitro, inactivation of RPA70A (by mutating the 2 conserved stacking aromatic residues) eliminated RPA binding to a single substrate (25). In contrast, similar inactivation in RPA70B only reduced the binding. In vivo, a single mutation of the conserved stacking aromatics (Phe-238 to Ala), which inactivates RPA70A, was lethal for yeast. In contrast, a mutation of structurally similar Trp-361 in RPA70B was not lethal (26).

Overall, our data support a sequential model of DNA binding in which the major pathway involves RPA70A providing the initial interaction with the 5′-end of ssDNA, followed by B, C, and D domains, which generates 5′ → 3′ polarity. We believe this pathway is ordered perforce by the linkage effect rather than by explicit cooperative interactions, i.e. if the initial interaction occurs with RPA70A, the DNA is held in close proximity and with the correct polarity to the adjacent RPA70B domain.

Linkage of the A and B domains ensures coordination of their DNA binding activities. The fact that the calculation of the linkage effect using a highly simplified model agrees with the observed increase in affinity for the linked relative to the isolated domains is consistent with the two domains functioning as independent domains. Results from experiments to test and refine this hypothesis will be reported on in due course.

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