Replication Protein A (RPA), the heterotrimeric single-stranded DNA (ssDNA)-binding protein of eukaryotes, contains four ssDNA binding domains (DBDs) within its two largest subunits, RPA1 and RPA2. We analyzed the contribution of the four DBDs to ssDNA binding affinity by assaying recombinant yeast RPA in which a single DBD (A, B, C, or D) was inactive. Inactivation was accomplished by mutating the two conserved aromatic stacking residues present in each DBD. Mutation of domain A had the most severe effect and eliminated binding to a short substrate such as (dT)12. RPA containing mutations in DBDs B and C bound to substrates (dT)12, 17, and 23 but with reduced affinity compared with wild type RPA. Mutation of DBD-D had little or no effect on the binding of RPA to these substrates. However, mutations in domain D did affect the binding to oligonucleotides larger than 23 nucleotides (nt). Protein-DNA cross-linking indicated that DBD-A (in RPA1) is essential for RPA1 to interact efficiently with substrates of 12 nt or less and that DBD-D (RPA2) interacts efficiently with oligonucleotides of 27 nt or larger. The data support a sequential model of binding in which DBD-A is responsible for the initial interaction with ssDNA, that domains A, B, and C (RPA1) contact 12–23 nt of ssDNA, and that DBD-D (RPA2) is needed for RPA to interact with substrates that are 23–27 nt in length.

Replication Protein A (RPA) is a single-stranded DNA (ssDNA)-binding protein that plays an essential role in DNA metabolism, including replication, repair, and recombination (1). Human RPA (hsRPA) is a multimeric complex of three subunits, 70 kDa (RPA1), 34 kDa (RPA2), and 11 kDa (RPA3), that binds to ssDNA with high affinity and binds poorly to double-stranded DNA and RNA (2–4). RPA has been identified in numerous species including the yeast Saccharomyces cerevisiae (scRPA), where it is a heterotrimeric complex of 69-, 36-, and 13-kDa subunits (5, 6). The genes encoding scRPA1–3 are referred to as scRPA1–3, respectively, and each gene is essential for viability (5, 7). Each subunit of RPA is also known to be required for SV40 DNA replication in vitro (8, 9).

The binding of RPA to ssDNA has been analyzed by a number of methods, and a consensus has emerged on the size of ssDNA occluded by a bound trimer. Cross-linking of hsRPA to ssDNA revealed an initially unstable 8-nt binding mode that resolves to a stable mode in which 30 nt are occluded (10, 11). A high affinity 30-nt binding mode was also obtained for hsRPA and scRPA using fluorescence quenching and electrophoretic mobility shift assay (12, 13). The binding site size for a number of other species of RPA has been reported, including Drosophila melanogaster (22 nt) (14), calf (20–25 nt) (15), and yeast (20–30 nt) (16). However, a 90-nt binding mode has been reported for scRPA using fluorescence quenching and electron microscopy (17).

The RPA1 subunit displays strong ssDNA binding on its own (6, 18). The structure of the central domain of hsRPA1 has been determined and shown to consist of two structurally similar ssDNA binding domains (DBDs), or OB-folds (oligonucleotide/oligosaccharide binding folds) (19). Single-stranded DNA binding by these domains (A and B) is accomplished by aromatic amino acid residues stacking with the individual bases of ssDNA and by hydrogen bonds between the protein and both the phosphate backbone and DNA bases. DBD-A and -B contact 3 nt each, with 2 nt between the two domains. The C-terminal domain of RPA1 (DBD-C) is a third ssDNA binding domain that requires zinc and is likely to contain another OB-fold (20, 21). RPA2 contains a fourth binding domain (DBD-D) with an OB-fold structure (22–24). These four DBDs display amino acid sequence similarity particularly with respect to the aromatic residues known to stack with the ssDNA bases (20). Structure/function analysis revealed that the N-terminal 18 kDa of RPA1 (RPA1N) is unlikely to play a role in ssDNA binding, as it is dispensable for SV40 DNA replication and has no significant binding activity (20, 25). In addition, there is currently no evidence that RPA3 binds ssDNA.

Our understanding of how these four DBDs contribute to the mechanism of ssDNA binding is incomplete. RPA1 is thought to account for most if not all of the heterotrimer’s binding affinity (25, 26), as the interaction of ssDNA with RPA2 is weak (22, 23), and the ssDNA binding by the RPA2/3 sub-complex is difficult to detect (27). However, the binding affinity of the RPA2/3 sub-complex is stimulated 100-fold when the N and C termini of RPA2 are truncated to produce a “core” domain bound to RPA3 (24). A direct comparison of binding by the isolated DBDs is difficult due to their insolubility (20, 22), and a systematic analysis of the role of the individual DBDs within the context of the heterotrimer is lacking. Models of ssDNA binding by the heterotrimer can be formulated based on the evidence that the DBD-A/B dimer interacts with 8 nt of ssDNA.
Plasmids used in this study

| Name       | Insert | Relevant amino acid changes | Vector     | Reference or source |
|------------|--------|----------------------------|------------|---------------------|
| pRF6       | RFA1   | RPA1: S293L,N294E,I424G     | pET11a     | This study          |
| pJM223     | RFA2   | None                        | pET11a     | 7                   |
| pJM329     | RFA3   | None                        | pET11a     | 45                  |
| pJM322     | RFA2,-3| None                        | pET11a     | This study          |
| pSAS106(WT)| RFA1,-2,-3|                           | pET11a     | This study          |
| pSAS109(A) | RFA1,-2,-3| RPA1: F238A,F269A           | pET11a     | This study          |
| pSAS112(B) | RFA1,-2,-3| RPA1: W360A,F385A           | pET11a     | This study          |
| pSAS115(C) | RFA1,-2,-3| RPA1: F537A,Y568A           | pET11a     | This study          |
| pSAS103(D) | RFA2   | RPA2: W101A,F143A           | pET11a     | This study          |
| pSAS103(A B)| RFA1,-2,-3| RPA1: F238A,F269A,W360A,F385A | pET11a     | This study          |
| pSJ105     | RFA1   | None                        | pET11a     | This study          |
| pJM136     | RFA1   | None                        | pRS413     | 22                  |
| pJM243     | RFA2   | None                        | pRS415     | 22                  |
| pSAS204    | RFA2   | RPA2: W101A,F143A           | pET11a     | This study          |
| pJM128     | None   | None                        | pET11a     | This study          |

The simplest model to account for most of the data is that the four DBDs collectively interact with 18–20 nt and, together with flanking domains, occlude a total of 30 nt of ssDNA (28).

To systematically analyze the role of the four DBDs, we asked how each domain contributes to the overall binding affinity of RPA. To accomplish this, we inactivated a single DBD within the context of the RPA heterotrimer and compared its binding affinity to that of wild type (wt) RPA. Thus, RPA containing an inactive domain A, B, C, or D was purified and bound to substrates of various size. Using a short substrate, such as (dT)12, no stable interaction could be detected with RPA containing inactive domain A (RPA-A-), whereas the $K_a$ for RPA-B- or RPA-C- was ~1/3 that of wild type RPA. The $K_a$ for RPA-D- was unaffected for substrates 12–23 nt in length but was 1/3 to 1/2 that of wild type RPA for substrates of 40 nt or longer. These data suggest that domains A, B, and C interact with 12 nt and that DBD-D interacts with ssDNA greater that 23 nt. This conclusion was confirmed by in vitro cross-linking of substrate DNA to RPA.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The plasmids used in this study are listed in Table I. To express recombinant RPA using the T7 RNA polymerase system, we constructed triple expression plasmids in which each of the RFA genes is driven by its own T7 promoter. The wt RPA triple expression plasmid, pSAS106, which was used as the parent vector of all RFA1 aromatic amino acid mutants, was constructed from three separate expression plasmids. The RFA1, RFA2, and RFA3 open reading frames were ligated into pET11a (29) on Nde/BamHI cassettes to create pRF6, pJM223, and pJM329, respectively. The RFA1 open reading frame in pRF6 was amplified from pJM136 (22), which lacks internal NdeI sites. In addition, polymerase chain reaction (PCR) amplification with Vent DNA polymerase, created pJM223, pJM329, pJM243 and pJM243 for domain D. The specific aromatic residues were unambiguously identified by alignment with the human sequences for which the crystal structures are known, including human DBD-C (19, 20, 30). The A, B, C, and C’-PCR products were digested with BglII and SacI, SalI and Asp718, or BstW1 and SacII, respectively. The digested DNA fragments were then ligated into unique BglII and XhoI, XhoI and Asp718, or Asp718 and SacII sites of pSAS106, respectively. The A B- double mutant was made by a three-way ligation between the digested A- and B- PCR fragments and pSAS106, digested with BglII and Asp718. The D-PCR fragment was isolated on a NdeI/BamHI cassette followed by ligation into pET11a. This plasmid, pSAS204, was subsequently digested with BglII and BamHI, and the released fragment was ligated into the BglII site of pJM128 to create the triple expression plasmid pSAS103. All constructs were sequenced to show that only the intended changes were made.

**Protein Expression and Purification**—Recombinant RPA proteins were expressed in the *Escherichia coli* strain BL21(DE3) essentially as described (29). Cells were grown in LB medium with 100 μg/ml ampicillin at 37°C until the absorbance at 600 nm was 0.5. The cultures were induced for 2 h by adding isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM. Cells were collected by centrifugation and resuspended in buffer B (25 mM HEPES (pH 7.5), 0.01% Nonidet P-40, 1 mM EDTA, 718, or 0.1 mM phenylmethylsulfonyl fluoride containing 100 mM NaCl). All subsequent steps were performed on ice or at 4°C. Samples were subjected to 3 freeze-thaw cycles and 8 sonication periods of 15 s each. The lysate was centrifuged at 12,000 × g, and the supernatant was collected at a 50-mlü Affi-Gel blue affinity resin (Bio-Rad). The column was washed sequentially with 3 column volumes of buffer B containing 500 mM NaCl and buffer B containing 0.5 mM NaSCN. The protein was then eluted with buffer B containing 1.5 mM NaSCN. Peak fractions identified by the Bradford analysis were pooled onto a 5-ml hydroxylapatite column (Bio-Rad) and washed sequentially with 15 ml of buffer B containing either 40 mM NaHPO4 (pH 7.5), 120 mM NaH2PO4 (pH 7.5), or 500 mM NaH2PO4 (pH 7.5). The protein eluted in the 120 mM NaH2PO4 wash. Peak fractions containing RPA were identified by SDS, 17% polyacrylamide gel electrophoresis (PAGE), pooled, and dialyzed in buffer A (same as buffer B above, except that 25 mM Tris-HCl (pH 7.5) was substituted for HEPES) containing 100 mM NaCl. The dialyzed fractions were loaded onto a 1-ml Mono Q column (Amersham Pharmacia Biotech) washed with 3 ml of buffer A containing 100 mM NaCl and eluted in a 10-ml linear gradient from 100 to 400 mM NaCl. Peak RPA fractions were identified by SDS-PAGE, pooled, and dialyzed with buffer A until the conductance was equivalent to buffer A plus 25 mM NaCl. The diluted sample was applied to a 2-ml phosphocellulose column (Whatman) and washed with 5 ml of buffer A containing 500 mM NaCl or 1 mM NaCl. The protein was eluted in the 500 mM NaCl wash. Samples from the fractions were resolved by SDS-PAGE and those containing highly purified RPA were pooled and dialyzed in buffer B containing 25 mM NaCl or buffer B containing no EDTA, 20 μM ZnSO4, and 25 mM NaCl. Protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard.

**Single-stranded DNA Binding and Denaturing Immunoprecipitation Assays**—The standard DNA binding reaction was performed in a total volume of 15 μl and contained the indicated purified protein samples from *E. coli*, 2 fmols of 3P-labeled oligonucleotide (dT)12, (dT)17,

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A. Bochkarev, personal communication.
ssDNA Binding Domains of RPA

**Mutant Forms of RPA** — To determine the contribution of the four DBDs of yeast RPA to ssDNA binding, we sought to assay RPA proteins in which a single DBD had been inactivated. The inactivation of these DBDs was accomplished by mutating residues critical for ssDNA binding. The crystal structure of hsRPA domains A and B bound to ssDNA identified a number of residues that make specific hydrophobic and hydrogen bond interactions with ssDNA (19). We focused on the two aromatic residues that make hydrophobic stacking interactions with the ssDNA bases for the following reasons. Hydrogen bonding between RPA and the DNA bases is dependent on the sequence of the substrate DNA (19), and the amino acid residues involved in these interactions are not conserved in all four DBDs. In contrast, the hydrophobic stacking interactions appear to be independent of DNA sequence, and the positions of the aromatic residues are conserved in all four DBDs (20). Thus, mutation of the two aromatic residues would be expected to have the same effect in each DBD, allowing us to compare the relative roles of the four DBDs in ssDNA binding. Effects due to DNA sequence heterogeneity were eliminated by the use of homopolymeric oligo(dT) as substrate.

To assay mutant RPA proteins we designed an expression plasmid in which a variety of mutations could be introduced into a single RPA subunit and co-expressed with the remaining two subunits. Expression in bacteria was essential, as two of the single amino acid replacements (F238A and F537A) were previously shown to be lethal in yeast (20). The schematic diagram presented in Fig. 1 illustrates that wt RPA was expressed from a plasmid in which each DBD of RPA1 is encoded by a unique cassette. In addition, each of the three genes is driven by its own T7 promoter (not shown). Amino acid sequence alignment was previously used to identify the aromatic amino acids in each DBD that are homologous to the stacking residues identified in the crystal structure of hsRPA domains A and B (19, 20). To express RPA with an inactive DBD these residues were mutated to alanine in pairs (Fig. 1).

After expression in *E. coli*, RPA was purified using affinity and ion exchange chromatography. SDS-PAGE analysis of the purified proteins indicated a purity of at least 95% (Fig. 2). Mutation of domain A appeared to cause a significant structural change in the protein as the bands corresponding to the RPA1 subunit in the A− and A+ mutants migrated somewhat slower than those of wt or other RPA mutants. This behavior may be related to the significance of this domain in mediating ssDNA binding (see below).

**RPA Activity and Electrophoretic Mobility Shift Assay** — An electrophoretic mobility shift assay was used to determine the ssDNA binding affinity of wt and mutant RPA. This assay is a sensitive method for the analysis of RPA-DNA interactions that uses nanomolar concentrations of RPA so that equilibrium binding conditions are achieved (12, 13). Before performing these assays, we determined the percentage of purified RPA in our preparations that was able to bind ssDNA. A constant amount of RPA was incubated with increasing amounts of radiolabeled (dT)30, and the DNA-protein complexes were sep-
Fig. 2. Purified RPA complexes. ScRPA proteins were expressed in E. coli and purified as described under “Experimental Procedures.” Two μg of wt RPA or the indicated mutant was resolved by SDS, 17% PAGE and visualized with Coomassie Blue. The positions of RPA1 (69 kDa), RPA2 (36 kDa), and RPA3 (13 kDa) are indicated. The molecular mass standards are indicated in kDa.

Fig. 3. Determining the fraction of active RPA. Panel A, 8.7 fmol of purified wt RPA was incubated with the indicated amount of [32P]-labeled (dT)30, and the reactions were resolved on a 6% nondenaturing polyacrylamide gel. The positions of singly liganded (S) and unbound (F) substrate are indicated. Panel B, the radioactivity corresponding to free DNA and protein-DNA complex in A was quantitated using liquid scintillation counting. The fraction of RPA in the bound form was then calculated and plotted as a function of input DNA.

ssDNA Binding Domains of RPA

method of inactivating the DBDs. Mutation of DBD-C compromises binding of the (dT)17 substrate, whereas the A-B mutation eliminates binding of (dT)17. Interestingly, the D- mutant appears to bind this substrate as well as wt RPA.

In the case of (dT)40, titration with wt RPA revealed a retarded band that was saturated at equimolar levels of RPA (Fig. 4B). At the highest wt RPA concentrations, a second more slowly migrating form appeared. We interpret this to be a multiply liganded complex as previously observed (13). A similar response was obtained with the C- and D- mutants, although the multiply liganded complex occurs with somewhat lower levels of RPA-D- than with wt RPA. In contrast, we observed only a singly liganded complex with the A-B+ mutant, which only appeared at high protein concentrations (Fig. 4B). We conclude that under these conditions (dT)40 is sufficiently large to accommodate two RPA complexes. Furthermore, although the A-B- mutant is unable to bind a substrate of 17 nt, it retains the ability to bind a substrate of 40 nt. This suggests that RPA contains DBDs in addition to A and B that only function with longer oligonucleotides.

The binding affinity of wt and mutant RPA was determined by calculating equilibrium binding constants for substrates of various lengths. The intensity of the signal corresponding to the free and bound DNA was quantitated using liquid scintillation counting. The fraction of RPA in the bound form was then calculated and plotted as a function of input DNA.

Based on this qualitative assay, we conclude that wt RPA binds (dT)17 as a singly liganded form and that saturated binding requires a molar excess of RPA over substrate. Furthermore, mutating the stacking residues appears to be an effective method of inactivating the DBDs. Mutation of DBD-C compromises binding of the (dT)17 substrate, whereas the A-B mutation eliminates binding of (dT)17. Interestingly, the D- mutant appears to bind this substrate as well as wt RPA.

In the case of (dT)40, titration with wt RPA revealed a retarded band that was saturated at equimolar levels of RPA (Fig. 4B). At the highest wt RPA concentrations, a second more slowly migrating form appeared. We interpret this to be a multiply liganded complex as previously observed (13). A similar response was obtained with the C- and D- mutants, although the multiply liganded complex occurs with somewhat lower levels of RPA-D- than with wt RPA. In contrast, we observed only a singly liganded complex with the A-B+ mutant, which only appeared at high protein concentrations (Fig. 4B). We conclude that under these conditions (dT)40 is sufficiently large to accommodate two RPA complexes. Furthermore, although the A-B- mutant is unable to bind a substrate of 17 nt, it retains the ability to bind a substrate of 40 nt. This suggests that RPA contains DBDs in addition to A and B that only function with longer oligonucleotides.

The binding affinity of wt and mutant RPA was determined by calculating equilibrium binding constants for substrates of various lengths. The intensity of the signal corresponding to the free and bound DNA was quantitated and fitted to the Langmuir equation. The values of the binding constants determined from these and other titrations are presented in Table II, whereas Fig. 5 summarizes the findings. For all proteins there was an increased binding affinity for (dT)60 compared with (dT)12 (Table II and Fig. 5). For example, the binding constants (Kd) for wt RPA ranged from 1.8 × 10⁸ M⁻¹ for (dT)12 to 2.3 × 10¹⁰ M⁻¹ for (dT)60. This effect was previously reported for hsRPA and is likely the result of an increase in the number of direct interactions between the DNA and RPA protein (13). In addition, the values for wt yeast RPA are similar to those
obtained for hsRPA (12, 13). In the case of the D− mutant, these values ranged from $1.8 \times 10^{10}$ M$^{-1}$ to $1.1 \times 10^{10}$ M$^{-1}$ (Table II). Therefore, the affinity of wt RPA for ssDNA is ~130-fold higher for a (dT)60 than a (dT)12, and the affinity of the D− mutant is 60-fold higher for a (dT)60 than a (dT)12. Interestingly, the binding constants of wt RPA and RPA-D− are essentially equal for (dT)12 through (dT)23, whereas the $K_a$ of RPA-D− is 1/3 that of wt for (dT)40. This suggests that RPA-D− is compromised only in its ability to bind substrates longer than 23 nt.

Among the RPA proteins with singly mutated DBDs, the most severe effect was observed with the A− mutant. No complex was detected using (dT)12 substrate, and although it bound longer substrates, its affinity was significantly reduced; binding to (dT)23 was 20-fold less than wt, whereas binding to (dT)60 was 7-fold less than wt. This suggests that although domain A plays an essential role in binding short ssDNAs, the additional DBDs assist in binding longer oligos. This idea is supported by the binding affinities of the B+ and C− mutants. Both the B− and C− mutants bound (dT)12, although their affinity was about one-third that of wt. Using the longer (dT)23 substrate, the binding affinity of the B− and C− mutants dropped to one-tenth that of wt. Using the longest substrate, (dT)60, the binding affinity of B− and C− returned to about one-fourth that of wt. This suggests that, in contrast to domain D, domains B and C play a significant role in binding 12–23-nt substrates.

Lastly, ssDNA binding by the A− B− mutant was severely impaired. It failed to bind (dT)12 and (dT)17 even at high protein concentrations and bound (dT)40 and (dT)60 30–40-fold less efficiently than wt. Taken together, these results suggest that domain A is important for binding events and is essential for (dT)12. Domains A and B are essential for binding (dT)17 and, together with domain C, bind substrates as small as (dT)12. DBD-D appears to play a role in binding substrates greater than 23 nt. An alternative explanation for the low binding affinity of some RPA mutants (e.g., RPA-A− B−) for short substrates, but not long ones, is that longer oligonucleotides provide an increased concentration of binding sites. To test this hypothesis, we incubated the A− B− mutant with increasing amounts of a short substrate and asked whether it could bind at high substrate concentrations. As shown in Fig. 6, binding by the A− B− mutant could be detected with 0.2 and 1.0 fmol of (dT)40 but could not be detected with a 100-fold excess of (dT)17 (Fig. 6). We conclude that the failure of these mutants to bind short oligonucleotides is due to the requirement for sequential binding by the four DBDs.

In Vitro Cross-linking of ssDNA to RPA—We have previously described a UV cross-linking assay to detect the interaction of ssDNA with RPA. In this assay, RPA is incubated with an equimolar amount of 32P-labeled ssDNA, cross-linked with UV light, and analyzed by SDS-PAGE (20, 22). Here, we searched for interactions with specific RPA subunits by denaturing the cross-linked products in the presence of SDS and immunoprecipitating RPA1 or RPA2 with specific antisera. The resulting antibody-RPA-DNA complex was collected on protein-A beads, resolved by SDS-PAGE, and visualized with phosphorimaging.

As shown in Fig. 7A, when RPA was cross-linked to small substrates such as (dT)8, a 70-kDa protein corresponding to the RPA1 subunit was labeled. In this experiment we also detected binding by RPA1 breakdown fragments that migrated at ~50 kDa. As the substrate size was increased from 8 to 96 nt, the intensity and size of this band increased. This increase in intensity reflects the increase in the $K_a$ of RPA as substrate size increases (Table II). At 52–96 nt, the signal intensifies and splits into two broad bands that likely correspond to the substrate bound to multiple RPA1 subunits. To observe the contribution of domain A to this reaction, we repeated the experiment with RPA-A− (Fig. 7B). Although the profile of signal is roughly the same, there was a significant reduction in the signal obtained with the 8-, 10-, and 12-nt substrates. Thus, as observed above, domain A is essential for binding small substrates. The binding of RPA-A− to substrates 17 nt or greater is reduced somewhat compared with wt; however, like wt, there was an increase in signal obtained with 52–96-nt substrates, and the appearance of two broad bands representing multiple RPA1 subunits bound to these substrates.

We next tested the interaction of RPA2 with ssDNA by UV-cross-linking. When wt RPA was incubated with substrates of 23 nt or less, the interactions between RPA2 and ssDNA were not detectable or were extremely weak (Fig. 8A). In contrast, when incubated with larger oligos, such as (dT)27 or (dT)30, a band migrating at about 45 kDa was easily detected. When incubated with larger oligos, such as (dT)60, a more intense band migrating at 56 kDa was detected. To confirm that these RPA2-labeled bands represent authentic interactions between ssDNA and RPA2, we repeated the experiment with D− mutant RPA (Fig. 8B). In this case the intensity of the bands corresponding to RPA2 bound to each oligonucleotide is
Table II

Binding properties of RPA mutants determined by mobility shift assay

| Aromatic residue mutants | Oligonucleotide length | $K_a \times 10^9$ |
|--------------------------|------------------------|------------------|
|                          | (dT)12                | (dT)17            | (dT)23          | (dT)40            | (dT)60            |
| WT                       | 0.18 ± 0.003          | 0.51 ± 0.011      | 2.6 ± 0.6       | 8.4 ± 0.7         | 23 ± 0.0          |
| A$^-$                    | 0.12 ± 0.0008         | 0.088 ± 0.0008    | 1.1 ± 0.001     | 0.34 ± 0.025      | 3.3 ± 0.4         |
| B$^-$                    | 0.65 ± 0.0008         | 0.035 ± 0.005     | 2.8 ± 0.3       | 6.7 ± 0.4         | 6.3 ± 0.4         |
| C$^-$                    | 0.18 ± 0.003          | 0.53 ± 0.00      | 1.7 ± 0.01      | 2.7 ± 0.0         | 11 ± 0.5          |
| D$^+$                    | 0.095 ± 0.0005        | 0.29 ± 0.009      | 0.61 ± 0.1      |                     |                   |
| A$^+$ B$^-$              | 0.7 ± 0.23            | 8.4 ± 4.8        | 25 ± 100        |                     |                   |

Fig. 5. Comparison of association constants ($K_a$). The apparent binding constants ($K_a$) of wt or the indicated mutant RPA are presented graphically as a function of substrate size. All data are taken from Table II.

Fig. 6. RPA-A–B$^-$ is unable to bind (dT)17 even at high concentration. Twenty ng of purified RPA-A–B$^-$ protein was incubated with the indicated amounts of labeled oligonucleotide. A, (dT)17. B, (dT)40. The reactions were resolved on a 6% polyacrylamide gel. A control reaction containing no protein was loaded in the first lane (–). Arrowheads indicate the position of the wells, some of which contain background signal. An arrow indicates the position of protein-DNA complexes.

Discussion

Although RPA is well studied, the functions of its individual subunits and multiple DBDs remain obscure. Specifically, it is not known what combination of subunits or DBDs account for the major ssDNA binding mode. The occluded binding site size of several species of RPA is between 22 and 30 nt (14–16), and experiments with both yeast and human RPA indicate that this 30-nt binding mode is achieved by RPA directly interacting with 20–30 nt of ssDNA (12, 13, 31). RPA1 has long been known to bind ssDNA on its own and accounts for a majority of RPA ssDNA binding activity (1). Binding by the central domain of RPA1 alone may account for the 30-nt binding mode, or additional DBDs may be required. It has also been proposed that RPA2 allows the complex to bind ssDNA in a higher order mode (22).

Recent structural analysis of RPA has provided sufficient details on the mechanism of ssDNA binding to allow us to test various models of RPA binding. The crystal structure of domains A and B has been determined in the presence (19) and absence of ssDNA (28). These domains, each comprising an OB-fold, reorient upon binding ssDNA and interact with a total of 8 nt. Although the solution structure of human RPA1N also revealed an OB-fold-like structure (21, 32), this domain is not known to bind ssDNA and may mediate interactions with other proteins (33–37). The C-terminal portion of RPA1 is a third ssDNA binding domain that binds zinc and appears to contain another OB-fold (DBD-C) (20, 21). Finally, structural analysis of a sub-complex consisting of the RPA2 core bound to RPA3 revealed OB-folds in each of these domains (30). However, only the fold in RPA2 (DBD-D) resembles domains A and B, and only RPA2 has been shown to bind ssDNA in vitro (22, 24, 30). Thus, RPA consists of six potential ssDNA binding domains, of which four are known to bind ssDNA.

To test the role of the four DBDs in ssDNA binding, we considered the following models. The first model suggests that RPA1 alone is responsible for the 30-nt binding mode. The fact that DBDs A, B, and C are required for stable binding to substrates as small as 12 nt makes this idea unlikely as an-other 18 nt need to be occluded. A second idea is that RPA2 interacts with 23–27 nt is inconsistent with this model. The third model proposes that the four known
DBDs are required for the stable 30-nt binding mode. A detailed version of this model has recently been presented in which domains A, B, and C align in a linear fashion and contact 13–15 nt of ssDNA. DBD-D is then proposed to align with these domains such that a total of 18–20 nt of ssDNA is contacted by RPA. Domains RPA1N and RPA3 are proposed to account for the observed occlusion of 30 nt (28).

As illustrated in Fig. 9, most of our data are compatible with this model. Based on the co-crystal structure of hsRPA bound to ssDNA (19), we indicate that domains A and B of scRPA bind 8–10 nt of ssDNA. Next, we determined that DBD-A is essential for RPA to interact with (dT)12 and that DBD-B and -C are required for full binding affinity to this substrate. The fact that mutation of DBD-D had no effect on the affinity of RPA for (dT)12 or (dT)17 and only a minor effect on (dT)23 indicates that domains A, B, and C alone are responsible for interacting with 12 to 23 nt. DBD-D becomes important for binding substrates that are 23–27 nt in length (Fig. 9). Based on these data, we suggest that DBD-D is required for the occluded 30-nt binding mode. In particular, the data presented here are consistent with recent studies on the accessibility of hsRPA to ssDNA overhangs on hairpin substrates. Our results indicate that scRPA requires a minimum of 23 nt to display interactions with all four DBDs. This result is consistent with the fact that 5’ or 3’ overhangs of 23 nt allow optimum binding by hsRPA (38). Furthermore, we conclude that scRPA1 requires a minimum of 12 nt for strong ssDNA binding. This value is very close to the 13-nt binding mode of hsRPA that appears to be mediated exclusively by hsRPA70 subunit (39). As mentioned above, it is reasonable to assume that the remaining domains, RPA1N and RPA3, account for the occlusion of 30 nt.

A mutational approach has previously been used to study the role of DBDs A and B in hsRPA (40). Walther et al. (40) conclude that mutating a single conserved aromatic residue of DBD-A or -B had a minimal effect on the binding of hsRPA to (dT)30, as the binding affinity of hsRPA containing the F238A mutation was 67% of wt (40). This effect is not specific for hsRPA, as we have observed that scRPA containing the F238A mutation binds (dT)23 at 80% of the wt affinity. In contrast, the results presented here indicate that double aromatic mutation has a more profound effect. The affinity of RPA-A− for (dT)23 or (dT)40 was 1/20 that of wt RPA (Table II). Furthermore, this defect was amplified when binding to smaller

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3 S. A. Bastin-Shanower and S. J. Brill, unpublished results.
strates was examined; binding of RPA-A to substrates such as (dT)12 was not detectable (Table II). We conclude that mutating both aromatic residues significantly reduces the activity of a single DBD. In addition, it is important to consider substrate size when determining the effects of these mutations, as some effects are masked by the activity of additional DBDs within the RPA complex.

The UV cross-linking assay used here revealed strong interactions with RPA2. Previously, this assay suggested that the binding of ssDNA by RPA2 occurred with low efficiency and that it could be stimulated by increased concentrations of NaCl (22). By including an immunoprecipitation step in the experiments described here, we have found that the interaction between ssDNA and RPA2 is more efficient than previously thought. This result is consistent with the fact that dimeric (DBD-D/RPA3) or trimeric (DBD-C/-D/RPA3) subcomplexes of hsRPA bind ssDNA with relatively high affinity (24). We suggest that it is inherently difficult to identify an interaction between RPA2 and ssDNA in the context of wt RPA because binding by RPA2 requires prior binding by the potent RPA1 subunit. As mentioned above, a second difficulty in identifying this interaction is that the substrates shorter than 23 nt are unable to make efficient contact with DBD-D.

The idea that RPA2 plays a role in the major ssDNA binding mode of RPA is surprising given that it contributes only a small amount to the overall binding affinity of RPA. However, it is possible that this contribution is significant in vivo given that deletion of RPA2 is a lethal event in yeast. Previous attempts to inactivate DBD-B by mutation of either single aromatic stacking residue alone revealed that these mutants were viable (20). But as mentioned above, it is possible that mutation of a single stacking residue has a negligible effect on the binding affinity of an individual DBD. It will be of interest to further investigate the in vivo significance of the ssDNA binding activity of RPA2 by creating the double stacking residue mutation in DBD-B in yeast. The importance of domains A and C is underscored by the fact that these are the only domains that contain uniquely essential stacking residues (20).

In light of the small contribution of RPA2 to ssDNA binding affinity, one might predict that it plays an alternative role in the cell. For example, it might control RPA cooperativity or its interaction with other proteins. This function may in turn be regulated by the cell cycle and DNA damage-dependent phosphorylation of RPA2 (41, 42). In light of the present results, it is not surprising that phosphorylation of RPA2 did not significantly affect the overall ssDNA binding activity of RPA (43). We have previously suggested that RPA2 might be involved in a higher order binding mode, as exemplified by the salt-dependent 65-nt binding mode of E. coli ssDNA-binding protein (22). Although the present study revealed no evidence of this, it is possible that RPA2 or perhaps RPA3 participates in higher order binding under alternative binding conditions. In support of this possibility, we note that RPA-ssDNA complexes have been observed by electron microscopy to undergo significant compaction at high salt (44). Further study will be required to test the role of alternative binding conditions on the mechanism of ssDNA binding by RPA.

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