The Phosphorylation Domain of the 32-kDa Subunit of Replication Protein A (RPA) Modulates RPA-DNA Interactions

EVIDENCE FOR AN INTERSUBUNIT INTERACTION

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Replication protein A (RPA) is a heterotrimeric (subunits of 70, 32, and 14 kDa) single-stranded DNA-binding protein that is required for DNA replication, recombination, and repair. The 40-residue N-terminal domain of the 32-kDa subunit of RPA (RPA32) becomes phosphorylated during S-phase and after DNA damage. Recently it has been shown that phosphorylation or the addition of negative charges to this N-terminal phosphorylation domain modulates RPA-protein interactions and increases the sensitivity of DNA damage. We found that addition of multiple negative charges to the N-terminal phosphorylation domain also caused a significant decrease in the ability of a mutant form of RPA to destabilize double-stranded (ds) DNA. Kinetic studies suggested that the addition of negative charges to the N-terminal phosphorylation domain caused defects in both complex formation (nucleation) and subsequent destabilization of dsDNA by RPA. We conclude that the N-terminal phosphorylation domain modulates RPA interactions with dsDNA. Similar changes in DNA interactions were observed with a mutant form of RPA in which the N-terminal domain of the 70-kDa subunit was deleted. This suggested a functional link between the N-terminal domains of the 70- and 32-kDa subunits of RPA. NMR experiments provided evidence for a direct interaction between the N-terminal domain of the 70-kDa subunit and the negatively charged N-terminal phosphorylation domain of RPA32. These findings suggest that phosphorylation causes a conformational change in the RPA complex that regulates RPA function.

Replication protein A (RPA) is a heterotrimeric (70-, 32-, and 14-kDa subunit) single-stranded DNA-binding protein that requires DNA replication, recombination, and repair (1, 2). RPA was first identified as a factor necessary for SV40 replication (3–5). Subsequently homologues have been identified in all eukaryotic cells examined (1, 6). Human RPA binds single-stranded (ss) DNA with high affinity (7) but low specificity and cooperativity (8, 9). RPA has also been shown to specifically interact with multiple proteins involved in DNA metabolism (1, 2).

RPA is composed of three subunits with multiple structurally related, functional domains (Fig. 1). The primary ssDNA binding activity is localized to the central region of the 70-kDa subunit (RPA70) (10). This region is composed of two structural domains called DNA binding domains (DBDs) A and B (Fig. 1A) (11, 12). Both domains form an oligosaccharide/oligonucleotide binding fold (OB-fold) that contains five β-strands arranged into a Greek-key β-barrel capped by an α-helix between the third and fourth strands (13). The N- and C-terminal domains of RPA70 are also composed of OB-folds. Structural studies by NMR have shown that the N-terminal 168 residues of RPA70 form two distinct domains (14). The first 108 residues form an OB-fold, defined as DBD F based on structural homology and DNA binding ability (15). The remaining 60 residues are a flexible linker to DNA binding domains A and B in RPA70. In addition to binding DNA, DBD F has also been shown to interact with various proteins involved in DNA metabolism (1). The C-terminal region of RPA70, DBD C, exhibits weak DNA binding and is necessary for heterotrimeric complex formation (16, 17). The OB-fold of DBD C has two unique characteristics, a three helical cap and a zinc ribbon motif (18). Two additional OB-fold domains are located in the 32- and 14-kDa subunits. The 32-kDa subunit has a central OB-fold domain, DBD D, that has weak DNA binding, flanked by the N-terminal phosphorylation domain and a C-terminal winged helix domain (19). The latter has been shown to interact with proteins involved in DNA repair (19). The 14-kDa subunit (RPA14) is also composed of an OB-fold that has been defined as DBD E although there is currently no evidence that this domain interacts with DNA. RPA14 is necessary for RPA complex formation (1, 2).

In addition to binding ssDNA and proteins involved in DNA metabolism RPA can destabilize double-stranded DNA (dsDNA) (20, 21). Although RPA binds dsDNA with low affinity, it is able to promote helix destabilization by stabilizing single-strand regions in dsDNA (20, 22). RPA destabilization of duplex DNA is functionally distinct from that of helicases, because ATP and Mg2+ are not required (20). Linearized SV40 dsDNA is preferentially denatured by RPA at internal regions rich in A-T pairs and at both ends (21). Although other ssDNA-binding proteins are capable of destabilizing dsDNA, RPA is more efficient than the ssDNA-binding proteins tested: Escherichia coli SSB, herpes simplex virus ICP8 protein, bacteriophage T4 gp32, and bacteriophage T7 gp2.5 (20, 21, 23). Association and helix destabilization reactions of RPA with duplex DNA.
these studies are shown to the mutants in relation to wild-type RPA determined previously (22) and in after DNA damage (*) (29) are indicated. The activities of each of the RPA70(113–441) (34).

Helix destabilization by RPA requires the core ssDNA binding regions of basic (blue) and negative (red) surface potential and neutral surface potential (white).

DNA and duplex DNA containing a central eight-nt ss bubble indicated that the denaturation of dsDNA occurs in at least two steps (16, 23). The two steps are (i) the formation of stable RPA/ssDNA with a small region of ssDNA (nucleation), followed by (ii) efficient strand separation. The destabilizing activity of RPA may play a role in the initiation of replication, as well as the denaturation of damaged DNA, in nucleotide excision repair (16).

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tum correlation NMR (HSQC). These findings suggest that there is a conformational change in RPA upon phosphorylation involving an intersubunit interaction between DBD F in RPA70 and the N-terminal phosphorylation domain of RPA32. This conformational change appears to regulate RPA function through modulation of RPA interactions with both proteins and DNA.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (6000 μCi/mmol) was purchased from Amer sham Biosciences. The 35-residue peptides, RPA32-WT and RPA32-Asp, corresponding to the wild-type or aspartic acid-substituted N-terminal phosphorylation domain of RPA32, respectively (Fig. 1), were from the Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University. Biotinylated dT30 for surface plasmon resonance was purchased through the University of Iowa DNA Core (Sigma Genosys).

Buffers—HI buffer contained 30 mM Hepes (diluted from 1 M stock at pH 7.8), 1 mM dithiothreitol, 0.25 mM EDTA, 0.5% (v/v) insolitol, and 0.01% (v/v) Nonidet P-40. 1× Tris borate/EDTA (1× TBE) gel buffer contained 89 mM Tris, 89 mM boric acid, and 2 mM EDTA.

DNA Templates and Manipulation—A 40-nt dsDNA fragment of the early palindromic SV40 origin was made for helix destabilizing studies by labeling an oligonucleotide (SV40 top, 5′-CTCCAAAAAGGCTCCTCTCAGATTGAGCTTC-3′) with [32P]ATP by T4 polynucleotide kinase (New England Biosystems) following the manufacturer’s recommendations. The labeled DNA was separated from free ATP with a P-30 Tris chromatography column (Bio-Rad) following the manufacturer’s specifications and annealed to equal molar concentrations of the complementary sequence (SV40 bottom, 5′-TCTGAGCTTATCCAGGAGTTGCTTTTGGAG-3′) to make a 40-nt double-stranded DNA substrate for helix destabilization assays. A double-stranded 40-nt substrate containing an eight-nt bubble was made by annealing SV40 top to SV40 bottom (5′-TCTGAGCTTATCCAGGAGTTGCTTTTGGAGGACAGGGGCTTTTGGAG-3′). The underlined portion indicates a region of non-complementary bases. Annealing reactions (10 mM Tris, pH 7.5, 50 mM NaCl, and 50 mM NaClO4) were placed in 100-μl PCO machine at 95 °C for 3 min ramped to 22 °C by a rate of 0.01 °C/s with a hold temperature of 4 °C. Annealing was monitored by 15% polyacrylamide gel electrophoresis (1× TBE). In all experiments, greater than 95% of labeled DNA was in double-stranded form.

Proteins—Wild-type RPA was purified as described (43). A form of human RPA that could not be phosphorylated, RPA32ΔS4, 8, 11–13, 23, 29, 33, 39A,21A (RPA32Δa10), and one that mimics the constitutively phosphorylated form, RPA32 S8, 11–13, 23, 29, 33D, T21D (RPA32ΔaS8), were created and purified as described (2). The deletions of DBD F (RPA70Δ1–168 (RPA70Δ168)) (17), DBD C (RPA70Δ42–416 (RPA70ΔC142)) (Fig. 1), and the phosphorylation domain (RPA32 Δ1–13, 23, 29, 33 of RPA32ΔS8 as described previously (43)) were expressed as described previously (14). The 35N-labeled RPA70Δ169–616 (DBD F) was made by transforming plasmid p11d-RPA70 Δ169–616 (44) into DE3 cells. The cells were grown in 5 g/liter Celltone®-N medium (Martek Biosciences Corp.) supplemented with 1 g/liter glucose, 1.5 g/liter K2HPO4, 1.4 g/liter KH2PO4, 1 g/liter MgSO4–7H2O, and 11 mg/liter CaCl2–2H2O (14). The protein was purified by standard RPA purification procedures as described previously (43).

Helix Destabilization Assay—The proteins were dialyzed against HI-30 (30 mM KCl). The extent of dialysis was monitored by solution conductivity. Helix destabilization assays were carried out as described previously (22). Briefly, 15-μl reactions with HI buffer containing 30 mM KCl, 2 μl radiolabeled DNA, increasing amounts of RPA (0–3160 fmol), and 50 μg/ml bovine serum albumin were incubated for 20 min at 25 °C. Reactions were terminated by adding SDS to a final concentration of 0.2% (to disrupt RPA-DNA complexes), followed by 4% glycerol and 0.01% bromphenol blue. The reaction products were separated on a 15% polyacrylamide gel (1× TBE) at 200 volts for 2.5 h. The gels were dried and a PhastSystem phosphorimager (Pharmacia-LKB) was used to identify the bands. The radioactivity in each band was quantified using a Packard Instant Imager. The amount of dsDNA (%) was plotted against the concentration of RPA and then analyzed by non-linear least squares fitting to a Langmuir binding equation using Nonlin (22, 45). Although the destabilization of DNA by RPA is not a single-stranded DNA binding reaction, the Langmuir binding equation was used to precisely determine the midpoint of the transition between dsDNA and ssDNA. The midpoint (given with units of m–1) of the fitted curves was used as a value for comparing the activities of RPA forms in this assay.

Time Course of Helix Destabilization—Saturating amounts of RPA (562 fmol for wild-type RPA, RPA32Δa10, and RPA32ΔS8) and 3160 fmol for RPA32Δa8, RPA70Δ168, and RPA70ΔC142 were combined with HI-30 to create a 120-μl reaction mixture. The DNA mixture consisted of 60 μl containing 50 μg/ml bovine serum albumin and 120 fmol of radiolabeled double-stranded 40-nt substrate or a double-stranded 40-nt substrate with an eight-nt bubble. The reaction mixture was incubated with the DNA mixture. Samples (15 μl) were removed at the indicated time points and immediately placed in microcentrifuge tubes with SDS (0.2% final concentration) to dissociate RPA-DNA complexes. The zero-min time point was made by adding the saturating amount of RPA to a mixture containing 2 fmol of DNA, 50 μg/ml bovine serum albumin, and 0.2% SDS in HI-30. The products were separated on a 15% polyacrylamide gel (1× TBE). The radioactivity in each time point was quantitated by the Packard Instant Imager and plotted as a function of time.

RPA70Δ169–616 Interactions with Peptides—The NMR data collection was performed as described previously (14). Lyophilized RPA32-WT peptide and RPA32-Asp peptide were dissolved in the same buffer as [315N]RPA70Δ169–616. Two-dimensional H–13C correlation maps were acquired on two identical [315N]RPA70Δ169–616 samples after addition stoichiometric and superstoichiometric amounts of either RPA32-WT or RPA32-Asp peptide to each RPA sample. Final spectra contained nearly a 20-fold excess of peptide relative to protein, and spectral changes were minimal in final spectra with RPA32-Asp, indicating an approach to saturation of peptide binding. Spectra were acquired at 750 MHz on a Varian Inova console, using water flip-back HSQC. We used a spectral width of 3000 Hz, 128 increments in the indirect dimension. Temperature was 25 °C.

Surface Plasmon Resonance—Interaction of RPA with ssDNA was monitored using a surface plasmon resonance biosensor instrument, BIAcore 3000. The streptavidin biosensor surface was prepared by manually injecting 5'-biotinylated dT30 diluted to 0.5 nM in 10 mM sodium acetate, pH 4.8, and 1.0 mM NaCl into the desired flow cell. Proteins were diluted in BHS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.005% polyaspartate-20) from BIAcore brought to 1 mM dithiothreitol. Protein (20 μl of 4 nM solution) was loaded by the injection option with the dissociation time of 500 s and a flow rate of 10 μl/min. Each experiment was repeated at least twice. Data were analyzed with the BIA Evaluation program and fit to a bimolecular Langmuir binding curve.

RESULTS

Role of N-terminal Phosphorylation Domain in RPA-DNA Interactions—RPA is composed of a high affinity DNA binding core (DBDs A and B) and three additional domains that can interact with DNA (DBDs F, C, and D; see Fig. 1). Mutations that decrease ssDNA binding also show defects in double-stranded DNA binding and helix destabilization activities (see Table I and Ref. 22). There have been several reports of phosphorylation modulating DNA interactions, but no consensus models have emerged for how RPA phosphorylation affects DNA interactions (see the Introduction). Our initial studies with RPA phosphorylated with CDK2 family kinases or DNA-dependent protein kinase (DNA-PK) suggested that phosphorylation had minimal effects on RPA binding to single-stranded oligonucleotides. To extend these observations we compared the ssDNA binding activity of various mutant forms of RPA including N-terminal phosphorylation domain mutants (Fig. 1).

We have shown that addition of negative charges to the N-terminal phosphorylation domain of RPA32 modulates RPA-protein interactions and that a mutant form, which has aspartic acid substitutions in the N-terminal phosphorylation domain of RPA32 (RPA32ΔAsp), has properties similar to those of phosphorylated RPA. Binding of the mutant forms of RPA to a 30-mer oligonucleotide was determined by surface plasmon resonance. The apparent binding constants for the phosphorylation domain mutants were the same as the wild-type RPA complex (Table I). Deletion or introduction of multiple point mutations of the N-terminal phosphorylation domain had no

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effect on the binding constant with oligo(dT)30. This is consistent with previous deletion analysis demonstrating that the core DNA binding domain of RPA70 (DBDs A and B) is necessary and sufficient for high affinity binding to oligonucleotides (10, 22).

Single-stranded oligonucleotides are the most simple form of DNA to which RPA can bind. In the cell most single-stranded DNA is part of partially duplex structures. Therefore, we next examined the ability of the phosphorylation domain mutants to destabilize a short DNA duplex. In this assay increasing concentrations of RPA are incubated with radiolabeled duplex DNA, and the relative amount of dsDNA and ssDNA is determined at each protein concentration. A radiolabeled 40-nt oligonucleotide from the SV40 early palindromic region annealed to its complementary strand was used as the substrate. This region of DNA has been shown to require only T antigen and RPA for denaturation during the early steps of SV40 initiation (46). Protein-DNA complexes were dissociated with SDS, and the products were separated on a 15% polyacrylamide gel. The amount of ssDNA and dsDNA in each reaction was quantitated, and the midpoint of the transition was determined. Deletion of the N-terminal phosphorylation domain (RPA-32Asp8) or introduction of multiple alanine mutations (RPA-32Ala10) had no effect on helix destabilization (Fig. 3A). In contrast, introduction of multiple aspartic acidic residues in the N-terminal phosphorylation domain caused a 4-fold increase in the amount of protein needed to destabilized 50% of the DNA template (Fig. 3A). This decrease in helix destabilization activity is unusual. Other forms of RPA have been shown to have decreased helix destabilization activity. For example, forms of RPA with mutations or deletions in the core DNA binding domain (DBDs A and B) or in the conserved zinc finger domain (DBD C) have decreased oligonucleotide binding and helix destabilization activity (22). However, in all but one case, these mutants also had decreased affinity for oligonucleotides (Table I; see also Ref. 22). The one exception to this general rule is RPA-70AN168, which has wild-type affinity for oligonucleotides but also shows an activity in helix destabilization one-quarter that of wild-type RPA (see Table I and Fig. 3A). We conclude that both RPA-32Asp8 and RPA-70AN168 have altered interactions with double-stranded or partially duplex DNA substrates that are not caused by differences in ssDNA binding activity.

**Helix-destabilizing Activities of RPA with an Eight-nt Bubble Substrate**—Helix destabilization by RPA appears to be a multi-step process in which there is first the formation of an RPA-DNA complex at a small region of ssDNA (nucleation) followed by efficient strand separation coupled to binding of additional RPA molecules. A pseudo-origin substrate with an eight-nt bubble in the center has been used to investigate the steps that are effected in these RPA mutants (46). The rate-limiting step is hypothesized to be the generation of a small region of ssDNA, because RPA binds rapidly and with high affinity to ssDNA. The decreased helix destabilization of RPA-32Asp8 and RPA-70AN168 could be because of deficiencies in either nucleation or subsequent strand separation steps. If the decreased helix destabilization activity of RPA-32Asp8 and RPA-70AN168 could be rescued by a substrate with an eight-nt bubble, it would suggest a defect in the nucleation step.

When helix destabilization was examined using an eight-nt...
bubble substrate, two levels of helix destabilization activity were observed. Both RPA/H1852832 and RPA/H1852832Ala10 had activity similar to wild-type RPA, although RPA/H1852832Ala10 consistently had a slightly lower activity (Fig. 3B). In contrast, RPA/H1852832Asp8 and RPA/H9004N168 had one-half the destabilization activity with the eight-nt bubble substrate compared with wild-type RPA (Fig. 3B). Thus, the presence of an eight-nt bubble partially restored helix destabilization activity observed for RPA/H1852832Asp8 and RPA/H70ΔN168 with dsDNA. This indicates that these forms of RPA have defects in both nucleation and strand separations steps of unwinding.

Kinetics of Helix Destabilization—To investigate the kinetics of the nucleation event, a time course of helix destabilization was examined. Saturating amounts of RPA were incubated under helix destabilization conditions, and 15-μl samples were removed at the indicated time points. The amount of ssDNA present at each time point was quantitated and plotted as a function of time.

Wild-type RPA, RPA/H32Ala10, and RPA/H32Δ33 all completed destabilization of both DNA substrates within 5 min (Fig. 4, A and B). The RPA/H32Asp8 mutant took 15 min longer to complete destabilization of the dsDNA substrate than wild-type RPA (Fig. 4A) but was still able to reach the same final level of destabilized DNA. In contrast, RPA/H32Asp8 required only 1 min for destabilization of the eight-nt bubble substrate. This indicates that RPA/H32Asp8 has a kinetic defect that affects the generation or recognition of a small region of ssDNA.

A striking result of these studies is that removing DBD F (RPA/H70ΔN168) or the addition of negative charges to the phosphorylation domain of RPA32 (RPA/H32Asp8) caused similar effects on RPA interactions with complex DNA substrates. Furthermore, the absence of an altered affinity to oligonucleotides and altered kinetics of helix destabilization in these mutants indicated that these mutations are acting through a mechanism distinct from those that affect ssDNA binding. These data suggest a link between DBD F and the N-terminal phosphorylation domain of RPA70.

Residue-specific Interactions Monitored by HSQC NMR—To test the intersubunit interaction hypothesis, peptides corresponding to the non-phosphorylated (RPA32-WT) and the negatively charged (RPA32-Asp) phosphorylation domain (Fig. 1)
were individually incubated with ^15^N-labeled RPA70Δ169–616 (DBD F; the first 168 residues of RPA70). HSQC NMR was used to monitor changes in the backbone of RPA70Δ169–616 at five different concentrations of peptide. The final difference in ^1H and ^15^N chemical shift change in DBD F residues (RPA70Δ169–616) upon addition of RPA32-WT (left) or RPA32-Asp peptide (right). (A) a bar graph of the ^1H chemical shift change in DBD F residues (RPA70Δ169–616) upon addition of RPA32-WT (left) or RPA32-Asp peptide (right). C, a ribbon representation, generated by Molscript (36), of the backbone topology for residues 1–114 of the DBD F based on the structure determined by Jacobs et al. (14) (Protein Data Bank number 1EWI). The backbone positions of residues that have ^1H and/or ^15^N chemical shift changes upon the addition of RPA32-Asp peptide (left) or ssDNA (right) (15) are colored red. The threshold used for coloring was one-half of the greatest chemical shift change; residues above threshold with RPA32-Asp peptide are 29–31, 42, 44–45, 55, 57, 80, and 92–94 and with DNA are 34, 35, 41–42, 59–62, 86, 89, 91, and 93 (15).

Fig. 6. Residues in the protein-DNA interaction domain perturbed by RPA32-Asp peptide. A, a bar graph of the ^1H chemical shift change in DBD F residues (RPA70Δ169–616) upon addition of RPA32-WT (left) or RPA32-Asp peptide (right). B, a bar graph of the ^15^N chemical shift change in DBD F residues (RPA70Δ169–616) upon addition of RPA32-WT (left) or RPA32-Asp peptide (right). C, a ribbon representation, generated by Molscript (36), of the backbone topology for residues 1–114 of the DBD F based on the structure determined by Jacobs et al. (14) (Protein Data Bank number 1EWI). The backbone positions of residues that have ^1H and/or ^15^N chemical shift changes upon the addition of RPA32-Asp peptide (left) or ssDNA (right) (15) are colored red. The threshold used for coloring was one-half of the greatest chemical shift change; residues above threshold with RPA32-Asp peptide are 29–31, 42, 44–45, 55, 57, 80, and 92–94 and with DNA are 34, 35, 41–42, 59–62, 86, 89, 91, and 93 (15).
which there are increased interactions between the N-terminal domains of RPA70 and RPA32.

**DISCUSSION**

Several lines of evidence now indicate that the phosphorylation domain of RPA32 modulates RPA activity. (i) RPA is phosphorylated in a cell cycle-dependent manner (26, 27). (ii) Cell extracts exposed to UV radiation that contain phosphorylated RPA are deficient in SV40 replication. Replication activity is restored with the addition of purified non-phosphorylated RPA (30). (iii) RPA phosphorylation modulates RPA-protein interactions. The addition of negative charges either by mutation or phosphorylation of the phosphorylation domain causes decreased interactions with T antigen and DNA polymerase α. The negatively charged phosphorylation domain also enhances RPA-p53 interactions (37). (iv) A form of RPA lacking the phosphorylation domain, RPA-32Δ33, was not able to support SV40 DNA replication after pretreatment with DNA-PK. Without pretreatment of DNA-PK, RPA-32Δ33 is able to support SV40 replication. This suggests that under some conditions the phosphorylation domain may be required to overcome inhibitory effects of DNA-PK on components of the replication machinery (34). (v) *Saccharomyces cerevisiae* with the serine/threonine to aspartic acid mutations in the phosphorylation domain as the only functional copy of RPA32 is hypersensitive to methyl methane sulfonate and hydroxyurea treatment indicating that DNA damage is not being repaired. (vi) Here we show that a negatively charged phosphorylation domain causes decreased helix destabilization, suggesting that RPA phosphorylation modulates RPA-DNA interactions. Recently Oakley et al. (37) have shown similar changes with dsDNA interactions using RPA phosphorylated in vivo.

There is one report in the literature that cdc2-phosphorylated calf thymus RPA stimulates helix destabilization (38). These findings are not consistent with those presented here or recent studies by Oakley et al. (37). They are also not consistent with a decrease in helix destabilization activity observed with RPA phosphorylated by DNA-PK (47). The disparity remains unresolved but could be because of differences in phosphorylation sites or differences in assay conditions.

**Helix Destabilization**—RPA efficiently destabilizes dsDNA (20, 21). The destabilizing reaction has been functionally separated into two steps. The first step, termed nucleation, is the generation of a small region of ssDNA, either by DNA breathing or RPA association, culminating with the stable binding of RPA. The second step is efficient strand separation. As the first step of helix destabilization includes the stable binding of RPA to a small region of ssDNA, RPA mutants that effect ssDNA binding also effect destabilization (Table I; 22). There is a direct correlation between ssDNA binding and helix destabilization. There are two exceptions to this generalization. RPA-70ΔN168 has wild-type ssDNA binding activity but decreased dsDNA binding and helix destabilization (22). In addition, the phosphorylation domain mutant, RPA-32Asp8, also showed wild-type ssDNA binding but decreased helix destabilization activity (Table I). The finding that separate mutations affecting either the basic cleft of DBD F (RPA-70ΔN168) or the RPA32 phosphorylation domain (RPA-32Asp8) do not affect ssDNA binding but do have similar effects on helix destabilization suggests a link between these two domains. We hypothesized a direct interaction between the phosphorylation domain and the basic cleft. This interaction may prevent the N-terminal region of RPA70 from contacting the DNA and contributing to destabilization. A direct interaction between the basic cleft of RPA70 and the negatively charged phosphorylation domain was observed by HSQC NMR experiments. A peptide corresponding to the negatively charged (RPA32-Asp) phosphorylation domain was found to interact with DBD F whereas a peptide corresponding to the native, unphosphorylated sequence did not (Fig. 6). Furthermore, the residues perturbed by the RPA32-Asp peptide were predominantly in the basic cleft and correlated well with residues that interact with ssDNA (15). These data support the hypothesis that the negatively charged phosphorylation domain of RPA32 interacts specifically with DBD F in RPA70.

**Model for RPA Intersubunit Interaction**—We have presented evidence for a specific intersubunit interaction between a negatively charged phosphorylation domain of RPA32 and the basic cleft in DBD F. The negative charges can be a result of phosphorylation or aspartic acid mutations as in RPA-32Asp8. Increased interactions between the phosphorylation domain and DBD F are likely to cause a global conformational change in RPA. These intersubunit interactions also probably keep DBD F from interacting with DNA and contributing to dsDNA destabilization (Fig. 4). This model provides a mechanism by which the phosphorylation domain can modulate RPA activity. This conformational switch is consistent with the finding that the phosphorylation domain affects RPA-protein interactions indirectly by regulating the contacts of DBD F with T antigen and DNA polymerase α (37). The proposed interactions are also consistent with recent evidence that DBD F rotates independently of DBD allowing domain switch to between conformations (15).

**The Basic Cleft and Phosphorylation Domain**—There are six residues, five arginines and one lysine, that form the basic cleft in DBD F of human RPA (Fig. 2). The charge of the basic cleft in human DBD F has been found to be conserved in other RPA homologues, with the exception of two eukaryotes that are missing the entire DBD F in *Crithidia fasiculata* (trypanosome), 23.9% identity; *Pyrococcus furiosus* (archaeae bacteria), 11.8% identity. The basic cleft is also not conserved in *Cryptosporidium parvum*, a protozoal parasite. We conclude that the basic cleft is evolutionarily conserved and present in all metazoans and yeast that have been examined.

Several mutations within DBD F have been characterized in yeast. Many of those with UV and methyl methane sulfonate hypersensitivity phenotypes map to regions within the basic cleft (48). This sensitivity to methyl methane sulfonate and UV is similar to the phenotype observed in RPA32 mutants with negatively charged N-terminal phosphorylation domains of RPA32. Decreased rates of recombination, double-strand break repair and HO-gene conversion are also associated with mutations near the basic cleft (48–51). In addition, some DBD F mutations are temperature-sensitive (49) and have defective DNA damage checkpoints (52, 53). Recently, *S. cerevisiae* RPA trimer containing the rfa1 mutant t11 (K45Q) has been expressed and found to have similar ssDNA binding affinity to wild-type *S. cerevisiae* RPA but impaired Rad51 displacement from ssDNA (54). The genetic data demonstrates that DBD F is playing a role in DNA metabolism, particularly DNA repair. The studies presented here show that mutations that alter the structure and charge distribution of the basic cleft or the addition of negative charges to the phosphorylation domain have similar effects on RPA interactions with dsDNA. These mutations may prevent the basic cleft from participating in RPA-protein or RPA-DNA interactions that are needed for an appropriate cellular response to DNA damage.

The serines and threonines in the phosphorylation domain of RPA32 are conserved to varying degrees in eukaryotes. The total number ranges from eight to 12 for most eukaryotes examined (data not shown). The two RPA homologues that have less than eight serine and threonine residues have the remaining serines and threonines in conserved sites. The major
exception to the high level of conservation is *Schizosaccharomyces pombe*, which has only three serine/threonines at non-conserved locations in its N-terminal phosphorylation domain. This suggests that *S. pombe* uses a different mechanism of regulation not involving a basic cleft. A homolog of RPA32 called RPA4 was found to be expressed in quiescent cells (55). RPA4 lacks four serines (S121, S23L, S29T, and S33K) and one threonine (T31D) found in RPA32. The absence of the serines in RPA4 may reflect the proliferation status of the cell types it is expressed in or that it is part of complexes that are regulated differently.

**Implications for RPA32 Phosphorylation**—The effect RPA phosphorylation has on DNA repair pathways is not known. Phosphorylated and non-phosphorylated forms of RPA are equally active in *in vitro* SV40 DNA replication and nucleotide excision repair assays (56). However, deletion of the phosphorylation domain and part or all of DBD F decreases the ability of RPA to support nucleotide excision repair (57). The phosphorylation state of RPA has no effect on RPA-xeroderma pigmentosum complementation group A (XPA) interactions (57). XPA has been shown to inhibit RPA-dependent separation of a 24-mer from M13 (58). This may be an example of a coordinated effort of phosphorylated RPA and XPA to limit the opening of DNA around the lesion site. It remains possible that phosphorylation of RPA may modulate RPA function in nucleotide excision repair *in vivo*. In addition, the effect of RPA phosphorylation on recombination and base excision repair remains to be determined. We have shown that negative charges in the N-terminal phosphorylation domain can modulate RPA-DNA interactions and present data that these negative charges promote an intersubunit interaction with the basic cleft of the N-terminal region of RPA70. This conformational change can modulate RPA70-mediated interactions with DNA and proteins and cause modulation of RPA activity.

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