Regulatory Functions of the N-terminal Domain of the 70-kDa Subunit of Replication Protein A (RPA)*

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Replication protein A (RPA) is the major single-stranded DNA-binding protein in eukaryotes. RPA is composed of three subunits of 70, 32, and 14 kDa. The N-terminal domain of the 70-kDa subunit (RPA70) has weak DNA binding activity, interacts with proteins, and is involved in cellular DNA damage response. To define the mechanism by which this domain regulates RPA function, we analyzed the function of RPA forms containing a deletion of the N terminus of RPA70 and mutations in the phosphorylation domain of RPA (N-terminal 40 amino acids of the 32-kDa subunit). Although each individual mutation has only modest effects on RPA activity, a form combining both phosphorylation mimetic mutations and a deletion of the N-terminal domain of RPA70 was found to have dramatically altered activity. This combined mutant was defective in binding to short single-stranded DNA oligonucleotides and had altered interactions with proteins that bind to the DNA-binding core of RPA70. These results indicate that in the absence of the N-terminal domain of RPA70, a negatively charged phosphorylation domain disrupts the activity of the core DNA-binding domain of RPA. We conclude that the N-terminal domain of RPA70 functions by interacting with the phosphorylation domain of the 32-kDa subunit and blocking undesirable interactions with the core DNA-binding domain of RPA. These studies indicate that RPA conformation is important for regulating RPA-DNA and RPA-protein interactions.

RPA is a heterotrimeric (70-, 32-, and 14-kDa subunits) protein composed of multiple structural domains that contain oligosaccharide/oligonucleotide binding folds (OB-fold). OB-folds are found in many ssDNA-binding proteins (16, 17) and in RPA are designated as DNA-binding domains (DBD) A–F (18, 19). A schematic of RPA is shown in Fig. 1. The 70-kDa subunit (RPA70) has four OB-fold domains (F, A, B, and C) connected by flexible, unstructured linkers. The OB-fold at the N terminus of RPA70, DBD F, contains a basic cleft that interacts with both ssDNA and proteins involved in DNA metabolism (20–23). Analyses of DBD F in yeast have suggested that this domain has a role in DNA repair and the cellular response to DNA damage (10, 24, 25). However, the molecular functions of DBD F remain poorly understood. DBD F is connected to the high affinity DNA-binding core of RPA (DBD A and DBD B) by a long, ~67-residue flexible linker (26, 27). DBD A and DBD B are both necessary for high affinity binding of the RPA complex (27–29). DBD C, at the C terminus of RPA70, is required for heterotrimeric complex formation and has also been shown to interact weakly with DNA (30–33). The 32-kDa (RPA32) and 14-kDa (RPA14) subunits each have one OB-fold domain called DBD D and DBD E, respectively, and are required for complex formation. DBD D interacts weakly with DNA and although DBD E of RPA14 was originally defined solely on structural similarity (1, 2), it has recently been shown to interact with single-stranded telomeric DNA sequences (34). RPA32 also contains an N-terminal phosphorylation domain and a C-terminal winged helix domain (35). The phosphorylation domain is flexible in solution as indicated by proteolytic and NMR analyses (35, 36).

RPA is phosphorylated during the cell cycle and in response to cellular DNA damage. Low levels of phosphorylation by the Cdk2 family of kinases occur in S phase and G2/M phase of cells undergoing normal growth (37). Hyperphosphorylation of RPA by the phosphatidylinositol 3-kinase family of kinases (including DNA-PK, ATM, and ATR) is observed after cellular DNA...
Phosphorylation causes several changes in the biochemical properties of the RPA complex. Phosphorylated RPA or RPA with phosphorylation mimetic mutations shows altered interactions with double-stranded DNA (62, 63). The domains of RPA essential for double-stranded DNA binding are the DNA-binding core (DBD A and B) and DBD F (64). In addition, multiple RPA-protein interactions are altered after phosphorylation (63, 65–67). The proteins affected by RPA phosphorylation also primarily interact with the N terminus of RPA70 (DBD F or DBD A). These findings suggest that phosphorylation induces a conformational change(s) in RPA involving both N-terminal domains of RPA32 and RPA70. Supporting this model is the finding that a phosphomimetic peptide has been shown to directly interact with DBD F (62). This type of interaction may be a general mechanism of RPA regulation as DBD F has also been shown to interact with peptides from the transactivation domains of p53 and ATRIP (23, 68). The phosphorylation domain of RPA32 has similar characteristics to these transactivation domains as they all are unstructured and negatively charged (when RPA32 is phosphorylated). Furthermore, RPA has partial sequence homology with the transactivation domain of p53 (RPA32, 28GSPAPSQA55, versus p53, 3LSPLPSQA55) (23). There is also evidence that phosphorylation may induce conformational changes within the binding core of RPA (DBD A and DBD B) after phosphorylation (69).

To obtain a better understanding of the role of DBD F and the structural and functional changes that occur after phosphorylation, we have carried out functional analysis of multiple RPA mutant forms of RPA. These RPA mutants combine a deletion of DBD F from RPA70 with mutations in the phosphorylation domain of RPA32. We show that combining a deletion of DBD F with a phosphorylation mimetic form of RPA32 resulted in a RPA complex that had dramatically altered interactions with both DNA and Rad51. These data support the model that phosphorylation induces changes in RPA conformation. These findings also suggest that DBD F has two regulatory functions: (i) it interacts with protein, DNA, and the negatively charged phosphorylation domain of RPA32 to modulate RPA function and (ii) it prevents the negatively charged phosphorylation domain from forming an dysfunctional RPA conformation. Our results show that the latter conformation causes dramatic changes in the activity of the RPA complex.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (6000 μCi/mmol) was purchased from Amersham Biosciences. Rad51 and the Rad51 primary rabbit antibody were gifts from the Radding laboratory (70). SV40 large T antigen (Tag) was purified as previously described (71). The monoclonal antibody 419 was used to detect SV40 large T antigen (72). The secondary antibodies mouse anti-rabbit and goat anti-rabbit, both with horseradish peroxidase conjugate, were purchased from Sigma. The phosphate-citrate buffer with sodium perborate capsules (0.05 mM phosphate citrate and 0.03% perborate at pH 5.0 in 100 ml of water) and o-phenylenediamine tablets (200 μmol/tablet) were also purchased from Sigma. The three oligonucleotides used in binding assays (dT30, dT20, and dT15) were purchased from 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% (w/v) inositol, 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-bp dsDNA fragment derived from the early palindrome region of the SV40 origin was made for helix destabilizing studies by labeling an oligonucleotide (SV40 top, 5′-CTCTAAAGAGCTTTTCACTACCTTCTGGAAATAGCTCA-3′) with [γ-32P]ATP by T4 polynucleotide kinase (New England Biosystems) following the manufacturer’s recommendations. The labeled DNA was separated from free ATP with a P30 Tris chromatography column (Bio-Rad) following the manufacturer’s specifications and annealed to equal molar concentrations of the complementary sequence (SV40 bottom: 5′-TCTGAGCTATTC-CAGAAGTAGTGAGGAGGCTTTTTGGAG-3′) to make a 40-nt double-stranded DNA substrate for helix destabilization assays. A double-stranded 40-nt substrate containing an 8-nt bubble was made by annealing SV40 top to SV40 bottom B (5′-TCTGAGCTATTC-CAGAAGTAGTGAGGAGGCTTTTTGGAG-3′). The underlined bases indicate the region of non-complementary bases. Annealing reactions (10 mM Tris–HCl (pH 7.5), 20 mM MgCl2, and 50 mM NaCl) were incubated in a Midwest Scientific PCR Sprint machine at 95 °C for 3 min followed by a transition to 22 °C by a rate of 0.01 °C/s with a final hold temperature of 4 °C. Annealing was monitored by gel purification in 15% polyacrylamide gel electrophoresis (1× TBE). In all experiments, greater than 95% of labeled DNA was in the double-stranded form.

Construction of RPA Mutants—Plasmids directing the expression of RPA70Δ1–168 (a deletion of DBD F [RPA70ΔF]) (31) and phosphorylation domain mutants (73) have been described previously. Plasmids with mutations in both RPA70 (a deletion of the N-terminal 168 residues of RPA70) and the phosphorylation domain (serine/threonine to alanine mutations in the phosphorylation domain or the deletion of the 33 N-terminal residues of RPA32) were created by digesting plasmids p11d-trPA70ΔN168 (31) and p11d-trPA32Δ10 and p11d-trPA32Δ1–33 with AatII and XmaI. The resulting fragments were ligated to create p11d-trPA70ΔN168,32Ala10 and p11dtrPA70ΔN168,32Δ33. A mutant that had a deletion of the N-terminal 168 residues of RPA70 and serine/threonine to asparagine mutations in the phosphorylation domain were created by digesting plasmids p11d-trPA70ΔN168 and p11d-trPA32ΔSp8 (73) with AatII and SmaI. The resulting fragments were ligated to create p11d-trPA70ΔN168,32Δ33 and p11d-trPA32ΔSp8. The three combination mutant proteins will be referred to as RPA70ΔF-32Ala, RPA70ΔF-32Δ33, and RPA70ΔF-32ΔSp8, respectively.

Proteins—Wild-type RPA, RPA phosphorylation domain mutants (RPA32548, 8, 11–13, 23, 29, 33, 39A, T21A (RPA32Ala), RPA3258, 11–13, 23, 29, 33, T21D (RPA32Asp), and RPA32Δ1–33 (RPA32Δ33)) and RPA forms with combinations of mutations (RPA70ΔF-32Ala, RPA70ΔF-32ΔSp8, and RPA70ΔF-32Δ33) were purified by standard RPA purification procedures as previously described (74) (Fig. 2). The deletion of DBD F (RPA70Δ1–168 (RPA70ΔF)) was purified as described (31). The RPA70 tandem domains RPA70(179–298:181–303)-His (AA-His) and RPA70(298–424:301–422)-His (BB-His) were purified as described (28). At least two preparations were made for all the proteins examined in these studies.

Gel Permeation Chromatography—Individual RPA forms (82 to 300 μg) were brought to a final volume of 200 μl with HI–200 mM KCl and 3 μl of acetone (included volume marker), filtered through a Nalgene 4-mm syringe filter (0.2 micron), and loaded onto a 24-ml Superose 6 HR10/30 column (GE Biosciences) equilibrated with HI–200 mM KCl. The protein was eluted at a flow rate of 0.5 ml/min and elution was monitored by UV readings using the AKTA–FPLC system from GE Biosciences. The elution volume of each protein was calculated using the Unicon Evaluation software (Amersham Biosciences). The Stokes radius of each protein was determined using the Porath Correlation with standard proteins.

Protein Activity—All analyses shown are based on total active protein present. RPA preparations have been reported to vary in activity. To ensure our comparisons were not due to differences in activity between preparations, the activity of all forms of RPA were analyzed by reverse binding assay (75). All of the forms of RPA used had activities above 70%. Thus the correction for activity was never more than 30% and was usually between 10 and 20%.

Gel Mobility Shift Assay—Gel mobility shift assays were performed as described previously (74, 76). Each reaction containing 15 μl total volume was incubated for 20 min at 25 °C. The reactions were then brought to a final concentration of 4% glycercol and 0.01% bromphenol blue and electrophoresed on a 1% agarose gel in 0.1× TAE buffer at 110 volts for 1.5 h. The loading buffer does not affect the binding of RPA (73). The gels were then dried on DE81 paper supported with blotting paper and radioactive bands were visualized by autoradiography. The radioactivity in each band was quantitated using a Packard Instant Imager. Binding isotherms were generated by plotting the fraction of free ssDNA versus protein concentration for each reaction. The intrinsic binding constants were calculated by nonlinear least squares fitting of the data to the Langmuir binding equation using KaleidaGraph (Synergy Software) (75).

Helix Destabilization and Double-stranded DNA Binding Assays—All 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 (64, 74). Briefly, 15-μl reactions with HI buffer containing 30 mM KCl, 2 fmol of 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 on Whatman 3MM chromatography paper and the radioactive bands visualized by autoradiography. The radioactivity in each band was quantitated using a Packard Instant Imager. The proportion of ssDNA remaining was plotted against the amount of protein and the midpoint of the transition between dsDNA and ssDNA fitting to the Langmuir binding equation (64). Although the destabilization of DNA by
Regulation of RPA

RPA is not a simple bimolecular binding reaction, 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. Double-stranded DNA binding assays were performed like helix destabilization assays except that reactions were terminated, separated, and analyzed as described for gel mobility shift assay.

**Enzyme-linked Immunoabsorbant Assay (ELISA)**—ELISA was used to examine interactions between purified proteins. All incubations were carried out at 25 °C. Wells in microtiter plates were coated with 1 µg of an RPA form in 50 µl of water (0.2 µM) and incubated for 1 h. Plates were washed with phosphate-buffered saline (PBS) with 0.2% Tween 20 three times to remove unbound protein. Plates were blocked with 300 µl of 5% milk in PBS for 10 min and washed. The indicated amount of Rad51, SV40 large T antigen, or bovine serum albumin in 50 µl of PBS with 5% milk was added to each well, incubated for 1 h, and washed. Primary antibodies for Rad51 or T antigen (diluted 1:1000 in PBS with 5% milk) were added to the plates, incubated for 30 min, and washed. Anti-rabbit IgG peroxidase conjugate in 50 µl of PBS with 5% milk was added to the plates, incubated for 30 min, and washed. Plates were developed using 200 µl of 0.8 mg/ml 0-phenylenediamine in 0.05 M phosphate buffer with 0.03% sodium perborate. OD450 was then quantitated after 20–60 min using a microtiter plate reader. In each experiment, backgrounds were determined for wild-type and all mutant forms of RPA using bovine serum albumin as the secondary protein. This was a control for nonspecific interactions with the antibodies. The data shown has these background values subtracted. In all assays the background values for all proteins were similar and close to zero.

**RESULTS**

**RPA Combination Mutants**—Three forms of RPA with mutations in the phosphorylation domain of RPA32 have been previously described and used to probe the role of RPA phosphorylation (62). These mutants included an unphosphorylatable form with all phosphorylatable serine/threonine residues mutated to alanine (RPA-32Ala), a phosphorylation mimetic form in which the serine/threonine residues known to be phosphorylated in vivo were replaced with aspartic acid residues (RPA-32Asp), and a form in which the phosphorylation domain was truncated so that the residues shown to be phosphorylated in vivo are deleted (RPA-32Δ33). The phosphorylation mimetic form has been shown to cause the same effects as phosphorylation on DNA interactions and protein interactions (62, 63, 73). In addition the phosphorylation mimetic form has been shown to have altered localization in human cells (44).

To explore the mechanism of RPA regulation after phosphorylation we combined each of the three phosphorylation domain mutants with a form of RPA70 missing DBD F. The three combinations of RPA-70ΔF-32Ala, RPA-70ΔF-32Asp, and RPA-70ΔF-32Δ33 are shown schematically in Fig. 1B. There was no detectable change in the expression or stability for each of these mutant RPA complexes. All were expressed at high levels in *Escherichia coli* and were purified at yields similar to wild-type RPA (data not shown). Fig. 2A shows the purified fractions of each of the combination mutants. To confirm the stability and obtain information on the solution form of the combination mutants, each individual mutant and the combination mutant proteins were analyzed by gel permeation chromatography. RPA-32Ala had a Stokes radius indistinguishable from wild-type RPA (50.1 ± 0.2 versus 49.7 ± 0.1 Å, respectively; Fig. 2B). However, deletion of the phosphorylation domain in RPA-32Δ33 or DBD F in RPA-70ΔF decreased the Stokes radii to 47.8 ± 0.4 and 43.3 ± 0.2 Å, respectively. The presence of the aspartic acids increased the Stokes radius slightly to 51.6 ± 0.5. The Stokes radii of the combination mutants, RPA-70ΔF-32Ala (43.1 ± 0.1 Å) and RPA-70ΔF-32Asp (44.0 ± 0.2 Å) were indistinguishable from RPA-70ΔF, whereas RPA-70ΔF-32Δ33 had a decreased Stokes radius (41.4 ± 0.4 Å). We conclude that all forms are heterotrimeric complexes in solution. The increase in Stokes radius observed for RPA-32Asp suggests that it may have an altered conformation in solution. This change is small compared with the overall solution structure of RPA and suggests either a small global change or a large but localized conformational change. The combination mutant RPA-70ΔF-32Asp does not show a similar change.
FIGURE 2. Purified forms of RPA. A, 2 μg of each of the indicated forms of RPA were separated by electrophoresis on an 8–14% SDS-PAGE gel and visualized by staining with silver nitrate. Positions of the molecular markers and the wild-type RPA subunits are shown on the left and right side of the panel, respectively. Lanes 1–3 are RPA-70ΔF-32Ala, RPA-70ΔF-32Asp, and RPA-70ΔF-32Δ33, respectively. Lane 4 is wild-type RPA. Note that the 14-kDa subunit of RPA migrates near the dye front and does not stain well. B, bar graphs summarize the Stokes radius of the indicated RPA forms. Gel permeation chromatography experiments were repeated at least 3 times. Error bars indicate standard deviation.

ssDNA Binding Activity—Each mutant was initially examined for ssDNA binding activity. Gel mobility shift assays were performed with each mutant using a 30-residue oligonucleotide substrate (dT₃₀). For all of the forms of RPA tested the free DNA disappeared at a similar concentration of protein (Fig. 3A). This indicates that RPA-70ΔF-32Ala, RPA-70ΔF-32Asp, and RPA-70ΔF-32Δ33 all had an affinity for ssDNA similar to wild-type RPA (Fig. 3A). This is consistent with the properties found previously for the single mutations, RPA-70ΔF and RPA-32Asp (see Table 1) (62). However, the signal observed for the RPA-70ΔF-32Asp•dT₃₀ complex was weak (Fig. 3A, summarized in left panel of C). The radiolabeled DNA in each lane was quantitated and remained constant throughout the titrations (data not shown). We conclude that the RPA-70ΔF-32Asp•dT₃₀ is less stable and was dissociating during electrophoresis. This suggests that the RPA-70ΔF-32Asp•dT₃₀ complex may have altered properties even though the protein appears to bind to ssDNA with high affinity.

Helix Destabilization Activity—We next examined the ability of the combined RPA mutants to destabilize a short DNA duplex. In this assay, increasing concentrations of RPA are incubated with a radiolabeled 40-bp double-stranded DNA fragment. RPA binding promotes the conversion of the dsDNA to ssDNA (64). Protein-DNA complexes are then dissociated by the addition of SDS and the DNA products separated on a 15% polyacrylamide gel. The amount of ssDNA and dsDNA present in each reaction was then quantitated and the midpoint of the transition determined. As predicted both RPA-70ΔF-32Ala and RPA-70ΔF-32Δ33 had a decreased ability to destabilize dsDNA compared with wild-type RPA (Fig. 4A, Table 2). The concentration of protein needed with these mutants to cause destabilization was similar to that needed for RPA-70ΔF. However, when RPA-70ΔF-32Asp was tested for helix destabilization activity, we found no detectable destabilization of the dsDNA substrate (Fig. 4A and B). Even using 10-fold higher concentrations of RPA-70ΔF-32Asp did not result in detectable destabilization (data not shown). These results indicated that even though RPA-70ΔF-32Asp has wild-type affinity for dT₃₀, this combination of mutations eliminates helix destabilization activity.

Helix destabilization by RPA is thought to be a multistep process that requires both a nucleation step in which RPA binds to transient single-stranded regions in the duplex DNA and a destabilization step (or steps) in which stable RPA binding converts the duplex to single-stranded DNA (62, 78). We have shown previously that RPA-70ΔF and RPA-32Asp are partially defective in both steps of this process. To gain a better understanding of the defect in the combination mutant RPA-70ΔF-32Asp, we also examined destabilization of a 40-bp substrate containing a central 8-nt bubble. It is thought that the single strand bubble provides an initial binding site for RPA that increases the rate of nucleation. RPA-70ΔF and RPA-32Asp show a decrease in destabilization activity relative to wild-type RPA similar to that reported previously (Fig. 4C, Table 2) (62). The combination mutants RPA-70ΔF-32Ala and RPA-70ΔF-32Δ33 showed destabilization activity similar to RPA-70ΔF and wild-type RPA, respectively (Fig. 4C, Table 2). This suggests that RPA-70ΔF-32Ala has defects in both nucleation and destabilization steps (Table 2). No helix destabilization activity was observed with RPA-70ΔF-32Asp using the 8-nt bubble substrate at any concentration of protein tested (Fig. 4C, Table 2). We conclude that RPA-ΔF-32Asp does not have detectable helix destabilization activity even when provided with a single-stranded nucleation point.

We also examined the binding activity of the combination mutants in a gel mobility shift assay using the 40-bp double-stranded oligonucleotide. This assay measures the initial binding step associated with helix destabilization but does not require complete unwinding of the DNA fragment. RPA-70ΔF-32Ala and RPA-70ΔF-32Δ33 both had reduced binding to the double-stranded oligonucleotide with affinities similar to that of RPA-32Asp (Fig. 4D). This reduction was similar to the helix destabilization activity observed (Fig. 4C). In contrast to the helix destabilization assays, RPA-70ΔF-32Asp was found to
bind to the double-stranded DNA. RPA-70ΔF-32Asp had an affinity that was an order of magnitude lower than RPA/H1852870/H9004F-32Ala and RPA/H1852870/H9004F-32Asp (Fig. 4D). When binding to the 8-nt bubble substrate was analyzed, RPA-70ΔF-32Asp had an affinity similar to RPA/H1852832Asp (data not shown). These findings demonstrate that RPA/H1852870/H9004F-32Asp can bind to duplex DNA or DNA containing an 8-nt bubble but is incapable of destabilizing duplex DNA. We conclude that this combination of mutations has a greater affect on the destabilizing steps than the nucleation step of this reaction.

Interactions with Short Oligonucleotides—Helix destabilization activity of RPA depends upon the DNA binding activities of DBD F and the DNA-binding core in RPA70 (64). There is no DBD F domain in the combined mutants. This suggests that the reduced destabilization activity of RPA-70ΔF-32Asp is probably caused by a change in activity of the DNA-binding core. Therefore we probed the effects of the combined mutants on the activity of the DNA-binding core. Structural analysis has shown that the core DNA-binding domains interact with 8 nucleotides of ssDNA (79). In addition, RPA exhibits substrate length-dependent binding with oligonucleotides shorter than 20 nt (75, 80). RPA binding to short oligonucleotides directly reflects interactions with the core DNA-binding domain, because the length of the oligonucleotide is sufficient to fill only the core interaction site and not other domains in RPA. Gel mobility shift assays were performed with dT20 (Fig. 3B) and dT15 (Fig. 3C, right panel). Wild-type RPA binds dT20 with high affinity. The binding constants of complexes containing the single domain mutations, RPA-70ΔF, RPA-32Ala, RPA-32Asp, and RPA-32Δ33, or the combined double deletion, RPA70ΔF-

![FIGURE 3. DNA binding properties of RPA mutants. Autoradiograms of representative gel mobility shift assays with dT30 (A) and dT20 (B). Increasing amounts of the indicated form of RPA were assayed as described under "Experimental Procedures." Protein used: A, 0, 0.01, 0.1, 0.18, 0.32, 0.56, 1, 3.2, 10, and 100 fmol; and B, 0, 1, 3.2, 10, 32, 100, 320, and 1000 fmol. C, bar graphs summarizing the apparent association constants determined for each RPA form analyzed for dT30, dT20, and dT15. Error bars indicate S.D. of values from 2 or more experiments.

| RPA form          | dT30 binding | Wild-type activity | dT20 binding | Wild-type activity | dT15 binding | Wild-type activity |
|-------------------|--------------|--------------------|--------------|--------------------|--------------|--------------------|
| RPA wild-type     | 145 ± 64     | 100%               | 31.6 ± 9.2   | 100%               | 2.7 ± 0.6    | 100%               |
| RPA-70ΔF          | 139 ± 2      | 96%                | 22.0 ± 6.2   | 70%                | 5.2 ± 0.6    | 192%               |
| RPA-32Ala         | 121 ± 44     | 84%                | 33.4 ± 3.5   | 106%               | 4.1 ± 0.2    | 152%               |
| RPA-32Asp         | 121 ± 44     | 84%                | 33.4 ± 3.5   | 106%               | 4.1 ± 0.2    | 152%               |
| RPA-32Δ33         | 146 ± 68     | 101%               | 6.5 ± 0.2    | 21%                | 0.49 ± 0.05  | 18%                |
| RPA70ΔF-32Ala     | 155 ± 7      | 107%               | 5.0 ± 0.4    | 16%                | 0.61 ± 0.16  | 23%                |
| RPA70ΔF-32Δ33     | 176 ± 65     | 121%               | 28.4 ± 4.8   | 90%                | 5.7 ± 0.9    | 210%               |

* Binding constants determined in Ref. 64.
32Δ33, all had affinities for dT_{20} similar to wild-type RPA (Fig. 3C; middle, see also gels in 3B). In contrast, both RPA70ΔF-32Ala and RPA70ΔF-32Asp had binding affinities ~20% of wild-type RPA (Table 1). In addition to the affinity being lower, the protein-DNA complexes formed with RPA70ΔF-32Ala and RPA70ΔF-32Asp appeared to be less stable based on the absence of well defined complex bands (compare autoradiograms in Fig. 3B). The decrease in band intensity is not due to the degradation of labeled DNA, because the total quantity of radioactive DNA in each lane remained constant and is comparable with the amount in the RPA wild-type reactions performed at the same time (data not shown).

Similar binding activities were observed in assays using dT_{15} as the substrate (Table 1). All forms of RPA had affinities similar to wild-type RPA except for RPA70ΔF-32Ala and RPA70ΔF-32Asp, which were decreased to ~20% that of wild-type RPA (Fig. 3C). We conclude that the addition of either multiple aspartic acid or alanine substitutions in the phosphorylation domain of RPA32 in the absence of DBD F results in altered interactions between the RPA70 high affinity binding core and short ssDNA. Thus either modified phosphorylation domain is capable of modulating DNA binding of the RPA complex and this regulation is normally prevented by a property of DBD F. The finding that both RPA70ΔF-32Asp and RPA70ΔF-32Ala have decreased binding to

![FIGURE 4. Helix destabilizing properties of RPA mutants. A, autoradiograms of representative helix destabilization assays. The gels are labeled on the left with the RPA form. The amount of protein added on each gel: 0, 1, 10, 32, 56, 100, 180, 320, 560, 1000, and 3200 fmol; the midpoint of each titration is indicated by an arrowhead at the bottom of each gel. B and C, bar graphs summarizing the average midpoint of helix destabilization for dsDNA (B) and the 8-nt bubble substrate (C). RPA70ΔF-32Asp has no detectable destabilization activity for either dsDNA or 8-nt bubble. D, bar graph summarizing the average apparent association constants determined for each form of RPA binding to dsDNA. Above each graph is a schematic of RPA destabilizing or binding the dsDNA substrate. Error bars indicate S.D. of values from 2 or more experiments.

![TABLE 2 Helix destabilization of RPA forms](https://www.jbc.org/content/283/31/21565)

| RPA form          | dsDNA helix destabilizing<sup>a</sup> | Wild-type activity | 8-nt bubble helix destabilizing<sup>b</sup> | Wild-type activity |
|-------------------|--------------------------------------|--------------------|---------------------------------------------|--------------------|
|                   | ×10<sup>−4</sup>, m<sup>−1</sup>         | %                 | ×10<sup>−4</sup>, m<sup>−1</sup>            | %                 |
| RPA wild-type     | 1.95 ± 0.18                           | 100                | 4.23 ± 0.83                                 | 100                |
| RPA70ΔF           | 0.46 ± 0.06<sup>c</sup>               | 28                 | 1.61 ± 0.18<sup>c</sup>                     | 38                 |
| RPA32Ala          | 1.59 ± 0.08<sup>c</sup>               | 96                 | 2.97 ± 0.37<sup>c</sup>                     | 70                 |
| RPA32Asp          | 0.40 ± 0.01<sup>c</sup>               | 24                 | 1.78 ± 0.67<sup>c</sup>                     | 42                 |
| RPA70ΔF-32Ala     | 1.98 ± 0.05<sup>c</sup>               | 119                | 3.58 ± 0.77<sup>c</sup>                     | 85                 |
| RPA70ΔF-32Asp     | 0.42 ± 0.07                            | 22                 | 1.91 ± 0.66                                 | 45                 |
| RPA70ΔF-32Ala     | 1.09 ± 0.18                            | 56                 | 4.68 ± 0.90                                 | 111                |
| RPA70ΔF-32Ala     | 0.42 ± 0.07                            | 22                 | 1.91 ± 0.66                                 | 45                 |
| RPA70ΔF-32Asp     | 1.09 ± 0.18                            | 56                 | 4.68 ± 0.90                                 | 111                |

<sup>a</sup> Helix destabilization of a 40-nt dsDNA substrate.

<sup>b</sup> Helix destabilization of a double-stranded 40-nt substrate containing an 8-nt bubble.

<sup>c</sup> Helix destabilization determined in Ref. 62.
dT_{30} and dT_{18}, but only RPA-70ΔF-32Ala is able to destabilize dsDNA substrates demonstrates that these two proteins have different but overlapping properties and there are at least two mechanisms by which the combined mutants are affecting RPA-DNA interactions.

**Phosphorylation Domain Interactions with RPA70—** A phosphomimetic peptide has been shown to directly interact with the basic cleft of DBD F (62). In RPA-70ΔF-32Asp, it is likely that similar direct interactions could occur between the negatively charged phosphorylation domain and other basic areas in RPA. For example, the core DNA-binding domains, DBD A and B, both have a number of basic residues on their surface (79). Therefore, the decreased binding observed with RPA-70ΔF-32Asp may be a result of the negatively charged phosphorylation domain interfering with DBD A and/or B interactions with short ssDNA. If this is the case, then RPA-70ΔF-32Asp might also show altered interactions with proteins that interact with DBD A and/or B. To test this hypothesis we analyzed interactions with two proteins previously mapped to interact with the core DNA-binding domain of RPA: SV40 large T antigen and Rad51 (73, 81–84).

We examined RPA-protein interactions using ELISA. In these assays, wild-type or mutant forms of RPA were adsorbed to a microtiter plate and interactions detected after incubation with varying amounts of either SV40 large T antigen or Rad51. To confirm which domains were important for these protein interactions, control assays were done with recombinant proteins containing two copies of DBD A (A1/A2-His) or two copies of DBD B (B1/B2-His) (28). Rad51 showed strong interactions with DBD A and minimal interactions with DBD B (Fig. 5A). SV40 T antigen interacted with both domains but showed greater interactions with DBD A than DBD B (Fig. 5B). These results demonstrate that DBD A is required for optimal Rad51 interactions and that both DBD A and DBD B participate in T antigen interactions.

As anticipated Rad51 and SV40 T antigen interacted with wild-type RPA (Fig. 5). Similarly, RPA forms with either a deletion of DBD F or mutations in the phosphorylation domain (RPA-70ΔF, RPA-32Ala, RPA-32Asp, and RPA-32Δ33) had interaction levels with Rad51 similar to wild-type RPA (Fig. 5A and data not shown). The combination mutant proteins, RPA-70ΔF-32Ala and RPA-70ΔF-32Δ33, also had wild-type interactions with Rad51 (Fig. 5A). In contrast, RPA-70ΔF-32Asp has decreased interactions with Rad51; the signal observed with RPA-70ΔF-32Asp was near that of B1/B2-His (Fig. 5A). RPA-70ΔF-32Ala and RPA-70ΔF-32Δ33 also interacted with T antigen at levels similar to wild-type RPA. RPA-70ΔF-32Asp showed a modest decrease in interactions with T antigen (Fig. 5B). This level of interaction, ~70% that of wild-type RPA, was similar to that observed with B1/B2-His. Thus, of the mutants tested only RPA-70ΔF-32Asp had dramatic changes in RPA-protein interactions. This suggests that in the absence of DBF, a negatively charged phosphorylation domain masks the Rad51 interaction site on DBD A. The modest decrease in interactions observed with RPA-70ΔF-32Asp and T antigen suggests that the interaction site in DBD A may be masked, and that it is the other sites of interaction in DBD B and RPA32 that allow continued binding.

**DISCUSSION**

Wild-type RPA binds very tightly to single-stranded oligonucleotides. The DNA-binding core (DBD A and DBD B) interacts directly with 8 to 10 nucleotides of DNA and is both necessary and sufficient for high affinity binding (27, 28, 85). Individually all of the mutations examined (RPA-70ΔF, RPA-32Ala, RPA-32Asp, and RPA-32Δ33) have DNA-binding activities similar to wild-type RPA with single-stranded oligonucleotides (Table 1). However, in this article we show that when DBD F is deleted, the addition of aspartic residues to the phosphorylation domain of RPA32 (RPA-70ΔF-32Asp) causes dramatic changes in the activity of the core DNA-binding domain: reduced binding to short oligonucleotides and no detectable helix destabilization activity. RPA-70ΔF-32Asp also showed a modest decrease in interactions with SV40 large T antigen and a dramatic decrease in interactions with Rad51. Because T antigen interacts with both DBD A and DBD B, whereas Rad51 only interacts with DBD A, the altered interactions suggest that RPA-70ΔF-32Asp has the most dramatic effects on DBD A. In the absence of DBD F, Ala mutations in the phosphorylation domain (RPA-70ΔF-32Ala) were also found to affect RPA interactions with short ssDNA. The simplest explanation of these observations is that in the absence of DBD F, the modifications to the phosphorylation domain are affecting the core DNA-binding domains thereby preventing normal function. The mechanism of this change could either be an induced confor-
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There is also little structural data in the literature on the conformation of phosphorylated RPA. Previously, we presented evidence for a specific intersubunit interaction between the basic cleft in DBD F and a negatively charged phosphorylation domain of RPA32 (62). In addition, Liu and co-workers (69) have suggested that phosphorylation of RPA results in an altered conformation of DBD B. The latter studies used intrinsic fluorescence and chemical modifications to examine the structures of in vitro phosphorylated RPA. Liu and co-workers (69) concluded that phosphorylation is directly modulating the conformation of DNA-binding domain B. However, the protein analyzed in those studies appears to have a large proportion of partially proteolyzed RPA70 (see Fig. 1 in Ref. 69). It is well established that the initial proteolytic cleavage of RPA70 occurs in the linker between DBD F and DBD A (33, 36, 91). So the proteolyzed, phosphorylated form of RPA analyzed by Liu and co-workers (69) should be structurally and functionally equivalent to the RPA-70ΔF-32Asp analyzed above. Indeed, the proteolyzed, phosphorylated RPA analyzed by Liu and co-workers (69) had decreased interactions with short ssDNA similar to that observed with RPA-70ΔF-32Asp.

The conformational changes observed by Liu and co-workers (69) confirm that there are structural changes in the DNA-binding core of RPA after phosphorylation and deletion of DBD F. Together these studies support a global conformational change in the structure of the DNA-binding core (DBD A and B). It is important to note that the dramatic functional changes observed in both studies only occurred in the absence of DBD F and in the presence of negative charges in the phosphorylation domain. We predict that these specific conformational changes in the DNA-binding core only occur when DBD F cannot exert its protective function.

Taken together previous studies and the data presented here indicate that alterations in the phosphorylation domain of RPA32 can modulate RPA structure and regulate multiple activities. We believe this regulation occurs in DNA damage-induced phosphorylated RPA. Fig. 7 summarizes the properties of the RPA phosphorylation mutants analyzed and shows a schematic to illustrate how conformational changes may regulate the activities of the mutant forms of RPA analyzed in these studies. In wild-type RPA, most DNA and protein interactions are mediated by RPA70. For example, the RPA70 DNA-binding core is responsible for high affinity binding (27, 28), DBD F is important for helix destabilization activity (62, 64), and DBD C appears to contribute to the stability of the RPA complex (27, 31). This is illustrated schematically in Fig. 7A by RPA interacting with a gray rectangle. In the absence of DBD F or if RPA32 is negatively charged, RPA has altered interactions between DBD F and DNA causing reduced helix destabilization activity. We propose that this is caused by the loss of the DBD F interactions with DNA (Fig. 7B, left side). In the case of the combination mutants analyzed in this study, RPA-70ΔF-32Δ33 has properties and presumably conformation(s) similar to RPA-70ΔF (Fig. 7B, top). RPA-70ΔF-32Asp has dramatically altered properties (Fig. 7C) suggesting this protein adopts a different conformation. Our data and that of Liu and co-workers (69) suggest that the negatively-charged phosphorylation domain is causing an altered conformation of the DNA-binding core (DBD A and B) and/or directly competing with proteins and short oligonucleotides to prevent/reduce their interactions with the core (Fig. 7B, bottom right).

The eight aspartic acid substitutions present in the 32Asp mutation represent a significant concentration of negative charges in the DNA-binding core (or a combination of both mechanisms). These findings also indicate that under normal conditions DBD F has a protective role that prevents the negatively charged phosphorylation domain from inhibiting the activity of the DNA-binding core.

**RPA Structure and the Phosphorylation Domain of RPA32—**

The three-dimensional structures of all the RPA domains have been determined as isolated domains or subcomplexes (35, 86–88). However, no global structure has been determined for RPA. Presumably this is because the structural domains of RPA are linked by flexible, and in most cases relatively long, polypeptide linkers. For example, the ~67 residue linker between DBD F and DBD A is intrinsically unstructured (26) and the phosphorylation domain normally exists in an extended, flexible conformation (36, 85, 89). Solution studies have indicated that except for the trimerization core of RPA (DBDs C, D, and E), the individual DBDs rotate independently and behave autonomously from each other in solution (20, 29, 90). These data indicate that RPA is a very flexible complex. This is emphasized in Fig. 6, which shows a hypothetical composite model of RPA in which all of the structured domains and linkers are shown at the same scale. (Note, Fig. 6 is not intended to represent the structure of RPA but rather to illustrate the relative amount of structured and flexible regions in the RPA complex.)

The apparent flexibility of RPA suggests that the phosphorylation domain could interact with any of the structured domains of RPA and that the RPA complex can adopt multiple conformations.
Regulation of RPA

Interaction with any (or all) of these basic areas to modulate RPA activity (see Fig. 6). Additional analysis will be needed to define the regulatory conformations of RPA after phosphorylation.

RPA-70ΔF-32Ala has a different combination of activities than either RPA-70ΔF-32A33 or RPA-70ΔF-32Asp (Fig. 7C). Alanine residues promote the formation of α helices and, therefore, the addition of 10 alanine residues to the phosphorylation domain would be predicted to convert this unstructured domain into an α-helix. Circular dichroism data from the RPA32-14 dimer containing the 10 alanine mutations supports this prediction (data not shown). We propose that it adopts a unique conformation (that is perhaps intermediate between RPA-70ΔF-32A33 and RPA-70ΔF-32Asp).

Implications for RPA32 Phosphorylation Domain Interactions—In full-length, phosphorylated RPA, it appears that there is an interaction between the negatively-charged phosphorylation domain and the basic cleft in DBD F that regulates RPA activity. The data presented above suggest that in the absence of DBD F, the phosphorylation domain could interact with other basic area(s) in RPA resulting in the masking of the DBD A protein interaction site. This model predicts that mutations in the basic cleft of DBD F would also be expected to alter RPA function. This has been observed in S. cerevisiae RPA: mutations mapping to regions near the basic cleft of DBD F were found to be hypersensitive to UV and methyl methanesulfonate in yeast (24). In addition, some mutations in the basic cleft of DBD F are temperature-sensitive, have defective DNA damage checkpoints, decreased rates of recombination, double-strand break repair, and/or HO-gene conversion (10, 24, 25, 93–95). Recently, one of the yeast RPA mutants, rfa1-t11 (containing a K45E mutation that reduces the positive charge of the basic cleft), was purified and found to have similar affinity for polynucleotides as wild-type yeast RPA, but was impaired displacing Rad51 from ssDNA (8). In human cells DBD F has been shown recently to not be necessary for cell growth but to play a role in the cellular response to DNA damage (77). Together, these data support a regulatory role for DBD F in the cell.

The importance of phosphorylation domain regulation by DBD F is also highlighted by analysis of protist RPA. RPA70 homologs identified in protists do not contain DBD F. The corresponding protist RPA32 genes either do not contain a phosphorylation domain or have a phosphorylation domain with a reduced number of phosphorylatable residues. This suggests that the two domains may have co-evolved. This is consistent with DBD F having a regulatory role relating to the function of the phosphorylation domain.

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