The RPA32 Subunit of Human Replication Protein A Contains a Single-stranded DNA-binding Domain

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Replication protein A (RPA) is a conserved nuclear single-stranded DNA (ssDNA)-binding protein. Human RPA (hRPA) comprises three subunits of approximately 70, 32, and 14 kDa (hRPA70, hRPA32, and hRPA14). RPA is known to bind ssDNA through two ssDNA-binding domains in the RPA70 subunit. Here, we demonstrate that the complex of hRPA32 and hRPA14 has an ssDNA-binding domain. Limited proteolysis of the hRPA14-32 complex defined a core dimer composed of the central region of hRPA32 (amino acids 43–171) and RPA14. The core dimer bound ssDNA with an affinity of approximately 10–50 μM, which is at least 100-fold more avid than the DNA-binding affinity of the intact dimer. Analysis of the predicted secondary structure of hRPA32 suggests that amino acids 63–150 of hRPA32 form an ssDNA-binding domain similar in structure to each of those in hRPA70. The complex of hRPA14 and hRPA32-(43–171) in turn formed a trimeric complex with the C-terminal region of hRPA70 (amino acids 436–616). The ssDNA-binding affinity of this trimeric complex was 3 to 5-fold higher than hRPA14-32-(43–171) alone, suggesting a role for the C terminus of hRPA70 in ssDNA binding.

Replication protein A (RPA) is a highly conserved eukaryotic ssDNA-binding protein that is involved in all aspects of DNA metabolism including replication, recombination, and repair (1). Human RPA (hRPA) is a heterotrimer with three subunits of approximately 70, 32, and 14 kDa (hRPA70, hRPA32, and hRPA14). In yeast, all three subunits are essential for viability (2). The diverse functions of RPA are mediated by its ssDNA binding activity, most of which has been attributed to the RPA70 subunit (1). The two smaller subunits, RPA32 and RPA14, form a stable complex (RPA14-32), but less is known about the role of these subunits in RPA function (3, 4).

The mechanism of ssDNA binding by hRPA involves at least two different binding modes, which are defined by the length of the interacting ssDNA. The first mode, which is characterized by an eight to ten nucleotide binding site, is quasi-stable (5–7). The second mode, which is associated with a binding state that occludes thirty nucleotides, is more stable (5, 6, 8, 9). The transition from the eight to the thirty nucleotide binding mode is thought to involve a large conformational change in the protein (6, 10) and is implicated in origin unwinding by SV40 large T antigen (11).

Most of the ssDNA binding activity in hRPA resides within the middle domain of hRPA70 (hRPA70-(181–422)) (10, 12, 13). Biochemical and structural studies of this domain (7, 14) revealed an ssDNA binding site of eight to ten nucleotides in length. The mechanism of the more stable binding mode remains unclear. Two formal possibilities exist. First, the 8–10-nucleotide ssDNA-binding domain in RPA70 may constitute the only ssDNA-binding domain in RPA, and other parts of RPA might preclude the binding of this domain to once every 30 nucleotides. Second, RPA may have additional ssDNA-binding domains that, together with hRPA70-(181–422), combine to form a 30-nucleotide binding site. Two lines of evidence support the latter possibility. First, the interaction of heterotrimeric hRPA with ssDNA is about 20-fold stronger than that of the isolated hRPA70-(181–422) domain, which points to direct interaction of the DNA with other parts of RPA (7). Second, RPA is predicted to have four ssDNA-binding domains on the basis of sequence homology (15); each of the RPA domains is thought to be structurally homologous to one subunit of the bacterial SSB homotetramer. Although the sequence homology is very weak, it has been in part supported by the structural analysis of the ssDNA-binding portion of RPA70 (14), which was confirmed to be composed of two identical SSB-like subdomains. The smaller two RPA subunits are also predicted to contain DNA binding activity, but there has been little direct biochemical support for this contention.

To explore the biochemical properties of the hRPA14-32 complex, we subjected the complex to limited proteolysis, co-expression analysis, and electrophoretic mobility shift assays. We identified ssDNA binding activity within the hRPA14-32 complex and further showed that the hRPA70 C-terminal domain also contributes in some way to ssDNA binding.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pET11a and pET15b were obtained from Novagen (Madison, WI). To construct plasmids pET15b-hRPA14, pET11a-hRPA32(43–171) and pET11a-hRPA70-(436–616), the fragments encoding hRPA14, hRPA32-(43–171), and hRPA70-(436–616) were generated by PCR using pET15b-hRPA14 and pET11d-hRPA70-(161–616) as templates. pET15b-hRPA14, pET11a-hRPA32(43–171) and pET11a-hRPA70-(161–616) was generously provided by Dr. M. O’Donnell (Rockefeller University) and Dr. M. Wold (University of Iowa), respectively.

The primers for hRPA14 and hRPA70-(436–616) were designed such that an NdeI site was added to the 5’-end and a stop codon with a BamHI site were added to the 3’-end. For hRPA32-(43–171), an NheI
site was added to the 5′-end, and a stop codon, an SpeI site, and an BamHI site was added to the 3′-end. The PCR products were digested with NdeI and BamHI for hRPA14 and hRPAT70-(436–616) or with NheI and BamHI for hRPA32-(43–171). The fragments were inserted into the pET15b vector for hRPA14 or pET11a vector for hRPA32 (for both hRPA32-(43–171) and hRPAT70-(436–616) between the corresponding NdeI/NheI and BamHI sites.

To construct the plasmid that co-expressed both hRPA14 and hRPAT70-(436–617) (pET15b-hRPAT70-(436–617)), an XbaI-HindIII fragment from pET11a-hRPAT70-(436–617) was inserted between the SpeI-HindIII sites of pET15b-hRPA14. To construct the plasmid that co-expressed hRPA14, hRPAT70-(436–616) and hRPAT70-(43–171) (pET15b-hRPAT70-(436–616), the XhoI-ExpI fragment from pET11a-hRPAT70-(436–616) was PCR amplified using a 5′-end primer that replaced the XhoI site with a BglII site. The standard T7 terminator primer (Novagen) containing an ExpI site was used for 3′-end. The PCR product encoding the hRPAT70-(436–616) was digested with BglII and ExpI and inserted into the pET15b-hRPAT70-(436–617) plasmid between the BamHI and ExpI sites.

The N terminus of hRPA14 in all the constructs was fused to a plasmid-encoded hexahistidine tag and a thrombin cleavage site. Digestion of the purified protein with thrombin resulted in the inclusion of the plasmid-encoded hexahistidine tag and a thrombin cleavage site. Digestion at the SpeI site resulted in the inclusion of the histidine tag and a thrombin cleavage site. Digestion of the purified protein with thrombin resulted in the inclusion of the plasmid-encoded hexahistidine tag and a thrombin cleavage site. Digestion at the SpeI site resulted in the inclusion of the histidine tag and a thrombin cleavage site.

**Protein Purification—**Bacterial BL21(DE3) pLyS cells (16) containing the RPA constructs were grown at 30 °C to an optical density of 0.6 (A∞00 nm) in 10 liters of Luria broth supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to 0.5 mM. Three hours after induction, cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5) and 1% sucrose, and frozen at −70 °C. Cells were thawed in the presence of EDTA, PMSF, benzamidine, dithiothreitol, and 0.2% Triton X-100. All subsequent steps were carried out at 4 °C. The cells were lysed by sonication and incubated for 0.5 h. The lysate was clarified by centrifugation at 70,000 × g for 30 min, and the salt concentration of the supernatant was adjusted to 500 mM NaCl. Nucleic acids and other negatively charged contaminants were removed from the lysate by adsorption to a 25-ml DE-52 column (Whatman) equilibrated in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 10% glycerol. The lysate was then loaded on an 15-ml metal chelating column (Novagen, Madison, WI) charged with 0.2 mM nickel sulfate and equilibrated in 50 mM Hepes (pH 7.4), 0.5 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mM benzamidine, and 5 mM imidazole (buffer A). The column was washed with 20 column volumes of buffer A, followed by five column volumes of buffer A containing 50 mM imidazole, and then developed with buffer A containing 300 mM imidazole. The eluate was dialyzed against 50 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, and 10 mM DTT and then incubated with bovine thrombin (5 units/mg of protein) for 2 h at 20 °C to remove the histidine fusion. After digestion, the protein was diluted with 50 mM Hepes (pH 7.4), 10% glycerol, and 1 mM DTT to final concentration of 50 mM NaCl and then loaded on a HPLC anion exchange chromatography column (Mono-Q, Pharmacia Biotech Inc.). The column was washed with 50 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, and 10 mM DTT and then developed with 10 ml of linear gradient from 50 to 1 M NaCl. The eluate was passed through a 5-ml metal chelating column (charged with 0.2 mM NiSO₄ and equilibrated in buffer A) to remove remaining contaminants. The flow-through was dialyzed against 1 mM Hepes, 50 mM NaCl, and 10 mM DTT. Protein concentrations were determined using the assay of Bradford (17).

**Limited Proteolysis Defines a Stable Core within the hRPA14-32 Heterotrimer—**The initial goal of this study was to characterize the complex of hRPA32 and hRPA14 and to investigate the role of these subunits in ssDNA binding. We first chose to define the regions of each subunit responsible for dimer formation. Our strategy was to use limited proteolysis of the intact hRPA14-32 dimer to identify a proteolytically resistant core of the dimer. To this end, the complex of hRPA14 with hRPA32 was expressed in Escherichia coli and purified to homogeneity with the aid of a hexahistidine tag on hRPA14. The purified complex was incubated with trypsin (a ratio of trypsin:RPA of 1:2000 by weight), and the extent of digestion monitored by gel electrophoresis (Fig. 1A). Under these conditions, the hRPA32 subunit was digested to a product of approximately 28 kDa in less than 5 min. This proteolytic domain has been previously characterized by Gomes and Wold (10) as an N-terminal 40 amino acid deletion. Concurrently more than 50% of the histidine-tagged hRPA14 was digested to a product of approximately 14 kDa. The 28 kDa fragment of hRPA32 was then digested to a 14-kDa fragment over the course of an hour (Fig. 1A). The two 14-kDa fragments, one derived from RPA32 and the other from RPA14, were resistant to further trypsin digestion even after 24 h (data not shown). The proteolytic products were analyzed by liquid chromatography. All attempts to resolve the two products by ion exchange chromatography were unsuccessful, suggesting that the two fragments formed a complex. The two 14-kDa fragments flowed through a Ni-chelate column, indicating that the histidine tag...
had been removed from hRPA14.

The molecular masses of the two fragments were determined by mass spectrometry. Two peaks with molecular mass of 14414 and 13704 Da were identified. Analysis of a predicted tryptic map revealed only one possible derivation for the two proteolytic products: a fragment from hRPA32, comprising amino acids 43–171 with a calculated molecular mass of 14416 Da, and an hRPA14 fragment, comprising full size hRPA14 (plus one methionine remaining from the fusion protein) with calculated molecular mass of 13707 Da. To confirm that these two polypeptides could form a complex when newly synthesized, they were co-expressed in E. coli and purified to homogeneity (Fig. 1B, lane B). As anticipated, they formed a stable dimer, termed hRPA14-(43–171). This complex was incubated with trypsin, but no further digestion was observed (data not shown).

The entire procedure was also performed using V8 protease, and similar results were obtained. Two V8-resistant fragments were identified as full size hRPA14 and the fragment 37–190 from hRPA32 (data not shown).

The C Terminus of hRPA70 Forms a Trimeric Complex with hRPA14-(43–171)—It was shown previously that the C-terminal part of hRPA70 is involved in heterotrimer formation (13, 18). We investigated further the interaction between the C-terminal fragment of hRPA70 and hRPA14-32. We tested if an hRPA70 fragment comprising amino acids 436–616 could interact with hRPA14-32. The N terminus of this C-terminal fragment begins just after the hRPA70 ssDNA-binding domain (amino acids 181–422) as defined by both biochemical and structural analysis. hRPA70-(436–616), hRPA32-(43–171), and hRPA14 were co-expressed in bacteria and purified to homogeneity (Fig. 1B, lane C). The trimeric complex co-purified through all chromatographic steps and was stable throughout the purification protocol under all salt concentrations from 50 to 750 mM.

Deletion of the N and C termini of RPA32 Uncovers ssDNA Binding Activity in hRPA14-32—With purified RPA subcomplexes in hand, we wished to test each for ssDNA-binding activity. Although most of the ssDNA-binding activity in RPA is thought to derive from RPA70, a role for the smaller subunits in ssDNA binding was postulated by Brill (15). However, ssDNA binding by the dimeric RPA14-32 complex has never been observed (1). We analyzed the hRPA14-32 complex for ssDNA binding activity using electrophoretic mobility gel shift assays. Increasing amounts of hRPA14-32 were incubated with 32P-end-labeled (dC)30 oligonucleotide and then resolved by gel electrophoresis (Fig. 2). In agreement with the results of other investigators, hRPA14-32 demonstrated little if any ssDNA-binding activity.

The deletion of the N and the C termini of hRPA32 increased ssDNA-binding activity dramatically. Whereas the binding of the intact dimer was virtually undetectable, the binding affinity of hRPA14-32-(43–171) for a (dC)30 oligonucleotide was about 10–50 μM. The binding was saturable (Fig. 2) and could be inhibited by the addition of unlabeled ssDNA (data not shown). The binding of hRPA14-32-(43–171) to DNA, although stronger than the intact hRPA14-32 dimer, was considerably weaker than that of the DNA-binding domains in hRPA70 (RPA70-(181–422) in Fig. 2), which when combined have an affinity of about 50–100 nM for a (dC)30 oligonucleotide.

The C-Terminal Fragment of hRPA70 Facilitates ssDNA Binding by hRPA14-32-(43–171)—We tested if the addition of the C-terminal fragment of RPA70 affected ssDNA binding. The purified trimeric complex hRPA14-32-(43–171)-70-(436–616) was shown to have 3–5-fold higher affinity for oligodeoxycytosine than did hRPA14-32-(43–171) (Fig. 2). The direct contribution of the RPA70-(436–616) domain to DNA binding activity could not be measured using biochemical assays because RPA70-(436–616), like RPA32, RPA70, and RPA32-(43–171), is insoluble when expressed individually. Again, it is important to note that although the ssDNA-binding affinity of the trimeric complex was higher than for the hRPA14-32-(43–171) dimer, it remained 100-fold lower than the ssDNA-binding affinity of hRPA70-(181–422).

Secondary Structure Prediction of the ssDNA-Binding Domain in hRPA32—Our DNA binding studies revealed an ssDNA-binding domain in the hRPA14-32 complex. We analyzed the sequence and secondary structure of these subunits for potential ssDNA-binding domains. Insofar as is known, all cellular ssDNA-binding proteins share a common three-dimensional fold (14), known as an OB-fold (19). The OB-fold has a characteristic secondary structure that can be predicted with high reliability by the secondary structure prediction program, PHD Predict Protein (20). This program accurately predicted the secondary structure of both ssDNA-binding domains in RPA70 and those in human mitochondrial SSB (21) and E. coli SSB (22).

Human RPA32 and RPA14 were analyzed with PHD Predict Protein. The program predicted that the secondary structure of the central domain of human RPA32 (amino acids 63–150) was consistent with an OB-fold topology (Fig. 3). Further analysis of this region revealed several regions of sequence homology that supports this prediction. First, RPA32 has several small peptide stretches that are similar to those known to be conserved in OB-fold proteins (Fig. 3) (23). Second, the two aromatic residues involved in the base stacking interaction within the subdomains A and B from ssDNA-binding domain of hRPA70 have equivalents in hRPA32-(63–150). Third, a D-X-T/S motif, which is conserved in the turn between the second and third β-strands in all RPA homologues (12, 13, 21, 22) was identified within hRPA32-(63–150).

A secondary structure prediction was calculated for hRPA14 and a C-terminal domain of hRPA70. This prediction did not reveal any structural motif that could be obviously interpreted as an OB-fold; the topological properties of these subunits must await structure determination.

DISCUSSION

In this manuscript, we demonstrated that the hRPA14-32 complex has intrinsic affinity for ssDNA, but this affinity is revealed only after deleting the C- and/or N-terminal domains of hRPA32. We also uncovered a potential role for the C terminus of hRPA70 in ssDNA binding; the addition of this domain to the smaller subunits increased binding affinity 3–5-fold. Our observations might provide an explanation for the structural
transitions observed for RPA during the ssDNA binding process (6, 10). We propose that hRPA initially binds to the ssDNA through the hRPA70-(181–422) fragment. This is a relatively high affinity interaction that corresponds to the 8–10-nucleotide binding mode. After the ssDNA binds to hRPA70-(181–422), the effective concentration of DNA is raised such that the lower affinity sites of hRPA32, and possibly RPA70, are filled. The binding to the lower affinity sites correlates with the appearance of the 30-nucleotide binding mode as well as an altered three-dimensional structure, as observed by electron microscopy (24). We suggest that the DNA-binding activity in the smaller subunits had escaped detection by all but cross-linking assays (15) because the-binding affinity of the smaller subunits for ssDNA is at least 100-fold lower than that of the largest subunit. Any influence of the smaller subunits on ssDNA binding by the RPA trimer would likely be masked by the activity of the largest subunit.

The discovery of a cryptic DNA binding activity in hRPA32 has regulatory implications. The inhibition of ssDNA binding by the N- and/or C-terminal domains of RPA32 suggests that the functional role of these domains is to down-regulate ssDNA binding by the RPA14/32 core. Although we have not yet determined which of the N- or C-terminal domains influences ssDNA binding activity, each has been implicated as a target for regulation. The N-terminal domain of RPA32 is a known substrate for the AT-kinase homologue in yeast, MEC1 (25), and for DNA-activated protein kinase in human cell extracts (26). Moreover, the phosphorylation of RPA32 is regulated in a cell cycle-dependent manner (27–29). Although studies have failed to uncover a functional role for the phosphorylation event, we suggest that the interpretation of these experiments warrant revisiting in light of the new model for RPA/ssDNA interaction. The C-terminal domain of hRPA32 is also a potential regulatory target. This domain interacts with DNA repair factors, including uracil-DNA glycosylase (30) and XPA (31, 32), and the RPA32-XPA interaction has been known to inhibit DNA replication in the SV40 system (33). It should also be noted that the yeast RPA14-32 dimer has also recently been shown to have ssDNA-binding activity. However, unlike for human RPA14-32, the intact yeast complex appears to have measurable activity. It remains to be established whether DNA-binding by the yeast complex is also modulated by neighboring domains or whether this regulatory phenomenon is specific for the human protein.

The concept that residues adjacent to an ssDNA-binding domain can regulate its activity is not unprecedented. The human transcriptional coactivator protein PC4 (or p15) contains cryptic ssDNA binding activity that was revealed only after removal of the N-terminal 61 amino acids (34). Interestingly, this N-terminal fragment is phosphorylated and this phosphorylation appears to regulate the activity of the protein (34).

A clearer picture is emerging for the domain structure of the RPA heterotrimer. The largest subunit is composed of four, and possibly five domains (10, 12, 35). The N-terminal domain extends from the N terminus to around residue 125, as defined by our limited proteolysis studies. This domain has been implicated in protein-protein interactions (10, 13, 18). The region from residue 125 to around residue 185 is unstructured, as determined by NMR analysis. The two DNA-binding domains in RPA70 are found from residues 191–291 and from residues 300 to 400 (14). The C-terminal region of RPA70 that extends from about residue 400 until the end contains one or more domains that mediate interactions with the RPA14-32 complex and might also play a direct role in DNA binding.

The RPA32 subunit comprises three domains, as defined both by limited proteolysis and by deletion analysis in yeast. The first domain extends from the N terminus to residue 40 and is phosphorylated. The middle domain extends from residues 40 to 170, interacts with the RPA14 subunit, and also likely contains an ssDNA-binding domain homologous to those in the RPA70 subunit. The C-terminal domain of RPA32 extends from residue 170 until the end and plays a role in protein-protein interaction (26, 30, 31). One or both of the RPA32 terminal domains may regulate ssDNA binding by that subunit. The RPA14 subunit was resistant to digestion with proteases and thus our studies did not shed light on the structure of this subunit. However, Brill and colleagues demonstrated that the N-terminal half of this subunit was not essential for yeast cell viability (15).

Bacterial SSB and human mitochondrial SSB are composed of four identical domains, each of which is structurally homolo-
gous to those in RPA70 (21, 22). Here we provide direct evidence for a third domain in RPA32 and weaker evidence for a fourth in the C terminus of RPA70. Our data support the model that there is a conservation of both domain and quaternary structure among all cellular SSBs, as first proposed by Brill (15).

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