Proteolytic Mapping of Human Replication Protein A: Evidence for Multiple Structural Domains and a Conformational Change upon Interaction with Single-Stranded DNA†

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Received November 10, 1995; Revised Manuscript Received March 4, 1996⊗

ABSTRACT: Replication protein A (RPA) is multisubunit single-stranded DNA-binding protein required for multiple processes in DNA metabolism including DNA replication, DNA repair, and recombination. Human RPA is a stable complex of three subunits of 70, 32, and 14 kDa (RPA70, RPA32, and RPA14, respectively). We examined the structure of both wild-type and mutant forms of human RPA by mapping sites sensitive to proteolytic cleavage. For all three subunits, only a subset of the possible protease cleavage sites was sensitive to digestion. RPA70 was cleaved into multiple fragments of defined lengths. RPA32 was cleaved rapidly to a ∼28-kDa polypeptide containing the C-terminus that was partially resistant to further digestion. RPA14 was refractory to digestion under the conditions used in these studies. The digestion properties of a complex of RPA32 and RPA14 were similar to those of the native heterotrimeric RPA complex, indicating that the structure of these subunits is similar in both complexes. Epitopes recognized by monoclonal antibodies to RPA70 were mapped, and this information was used to determine the position of individual cleavage events. These studies suggest that RPA70 is composed of at least two structural domains: an ∼18-kDa N-terminal domain and a ∼52-kDa C-terminal domain. The N-terminus of RPA70 was not required for single-stranded DNA-binding activity. Multiple changes in the digestion pattern were observed when RPA bound single-stranded DNA: degradation of the ∼52-kDa domain of RPA70 was inhibited while proteolysis of RPA32 was stimulated. These data indicate that RPA undergoes a conformational change upon binding to single-stranded DNA.

Replication protein A (RPA,[1] also known as human SSB and replication factor A) is a heterotrimeric single-stranded DNA-binding protein composed of subunits of 70, 32, and 14 kDa (Wold & Kelly, 1988; Fairman & Stillman, 1988). It is essential for DNA replication, DNA repair, and recombination (Wobbe et al., 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988; Coverley et al., 1991, 1992; Heyer et al., 1990; Moore et al., 1991). RPA homologues have been found in all eukaryotes examined (Brill & Stillman, 1989; Brown et al., 1992; Mitsis et al., 1993; Adachi & Laemmli, 1992; Atrazhev et al., 1992; Georgaki et al., 1992; Brown et al., 1992; Fang & Newport, 1993). This high level of homology suggests that the general structure and functions of RPA may be conserved throughout eukaryotes. Human RPA (hRPA) binds with high affinity to single-stranded DNA (ssDNA) (Wobbe et al., 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988; Kim et al., 1992) and interacts specifically with several proteins required for DNA replication including SV40 T antigen and DNA polymerase alpha/primase complex (Dornreiter et al., 1992). RPA also interacts with proteins involved in DNA repair such as XPA and XPG (Matsuda et al., 1995; He et al., 1995), transcriptional activators (He et al., 1993; Li & Botchan, 1993), and the cell regulator p53 (Li & Botchan, 1993; He et al., 1993; Dutta et al., 1993). The role of RPA−protein interactions is not understood; however, at least some of these interactions appear to be essential for RPA function (Melendy & Stillman, 1993; Lee & Kim, 1995; Santocanale et al., 1995). RPA also binds specifically to double-stranded DNA and may regulate gene expression (Luche et al., 1993; Singh & Samson, 1995).

There is strong biochemical evidence demonstrating that all three subunits of RPA are required for function (Kenny et al., 1990; Erdile et al., 1990, 1991; Umbricht et al., 1993; Henricksen et al., 1994; Gomes & Wold, 1995; Lee & Kim, 1995). In addition, in yeast, the genes for all three subunits of RPA are essential for viability (Heyer et al., 1990; Brill & Stillman, 1991). However, currently the precise roles of the individual subunits of RPA remain unknown. The 70-kDa subunit (RPA70) has intrinsic DNA-binding activity (Wold et al., 1989; Kenny et al., 1990; Gomes & Wold, 1995) and is involved in specific protein−protein interactions (Dornreiter et al., 1992; He et al., 1993; Dutta et al., 1993). Residues 1−441 of human RPA70 have been shown to be required for DNA-binding activity, and the C-terminus of RPA70 is necessary for stable interactions with the other two subunits of RPA (Gomes & Wold, 1995). The specific role of the 32- and 14-kDa subunits of RPA (RPA32 and RPA14, respectively) is unknown. These two subunits form

† This work is supported by Public Health Service Grant GM44721 from the General Medicine Institute.

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⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

[1] Abbreviations: RPA, replication protein A; hRPA, human replication protein A; rhRPA, recombinant human replication protein A; rhRPA·70N168, recombinant hRPA·70Δ1−168; ΔC442, RPA70ΔC442−616; Δ1−33, rhRPA·32Δ1−33; rhRPA·32Δ1−33, recombinant human RPA·32Δ1−33; rhRPA32·14, recombinant human 32−14 subcomplex; ssDNA, single-stranded DNA; RPA70, 70-kDa subunit of RPA; RPA32, 32-kDa subunit of RPA; RPA14, 14-kDa subunit of RPA; oligo(dT)$_{30}$, oligodeoxythymidine 30 residues in length; mab70A, monoclonal antibody αSSB70A; mab70C, monoclonal antibody αSSB70C; mab81, monoclonal antibody 81; mabRPA9, monoclonal antibody RPA9; SDS-PAGE, SDS−polyacrylamide gel.

S0006-2960(95)02699-7 CCC: $12.00

a stable structure in solution (Henricksen et al., 1994; Stigger et al., 1994) and appear to be important for the formation of the heterotrimeric RPA complex (Henricksen et al., 1994). In addition, RPA32 is phosphorylated in a cell-cycle-dependent manner (Din et al., 1990; Dutta et al., 1991; Fotedar & Roberts, 1992; Dutta & Stillman, 1992) and may participate in specific protein−protein interactions (Dornreiter et al., 1992; Lee & Kim, 1995).

Currently little is known about the structure of RPA. The hRPA complex is very stable in solution (Wold & Kelly, 1988; Fairman & Stillman, 1988). Hydrodynamic analysis indicates that hRPA is a heterotrimer with 1:1:1 stoichiometry (Kenny et al., 1990; Kim et al., 1994; Mitsis et al., 1993; Alani et al., 1992). In these studies, we examined the structure of hRPA using limited proteolysis. Cleavage of a protein by a protease requires direct interactions between the protease and individual peptide bonds. Thus, proteolytic cleavage provides a direct probe of protein conformation (Price & Johnson, 1989). Regions of proteins accessible to proteases most often occur in extended regions between domains or in loops exposed on the surface of a protein (Price & Johnson, 1989). Therefore, mapping protease sensitive sites provides information about the location of structural domains. Partial proteolysis has been used by many authors to identify protein domains, and it has been possible with well characterized proteins to use proteolytic footprinting to map protein domains involved in macromolecular interactions (Heyduk & Heyduk, 1994) and to quantitate structural changes occurring upon ligand binding (Pedigo & Shea, 1995). The studies presented here describe the initial proteolytic mapping of the 110-kDa hRPA complex. We treated RPA with two specific proteases (trypsin and chymotrypsin) and one nonspecific protease (bromelain). The specific sites of cleavage were mapped on both wild-type RPA and RPA deletion mutants using immunoblotting with monoclonal antibodies. These studies indicate that both RPA70 and RPA32 are composed of at least two distinct structural domains. In the presence of ssDNA, the proteolytic sensitivity of the RPA complex changed dramatically, indicating that RPA undergoes a conformational change upon binding to ssDNA.

EXPERIMENTAL PROCEDURES

Materials. All reagents used were of the highest purity commercially available. Restriction endonucleases, T4 DNA polymerase, polynucleotide kinase, and Klenow fragment were purchased from New England BioLabs and Life Technologies, Inc. [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]-ATP (4500 Ci/mmol) were obtained from ICN. Trypsin, chymotrypsin A_4, and bromelain were obtained from Boehringer Mannheim. Trypsin stock was 0.5 or 2 mg/mL in 10 mM HCl with 10% (mol/mol) tosyl-L-phenylalanine-chloromethyl ketone. Chymotrypsin stock was 1 mg/mL in 10 mM HCl. Bromelain was purchased as a concentrated solution (5 mg/mL) in 3.2 M ammonium sulfate. For each proteolysis experiment a fresh protease solution of 2−50 ng/μL was made by diluting stock solutions with HI buffer. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer model 380B by the DNA Core Facility at the University of Iowa. *Escherichia coli* DH5α was obtained from Life Technologies, Inc. *E. coli* expression strain BL21(DE3) was obtained from W. Studier (Studier et al., 1990).

HI buffer contained 30 mM HEPES (diluted from a 1 M stock at pH 7.8), 1 mM dithiothreitol, 0.25 mM EDTA, 0.25% (w/v) inositol, and 0.01% (v/v) Nonidet-P40. HI was supplemented with different concentrations of salt as indicated in the text. 1× Tris-Acetate/EDTA (TAE) gel buffer contained 40 mM Tris acetate and 2 mM EDTA, pH 8.5 (Ausubel et al., 1989). Sample loading buffer contained 4% ficoll, 2% SDS, 10 mM Tris-Cl (pH 6.8), and 10 mM dithiothreitol.

Expression plasmid p3a-RPA32 (Henricksen et al., 1994) contains a T7 RNA polymerase promoter followed by the cDNA sequence for the 32-kDa subunit of hRPA. p11d-tRPA (Henricksen et al., 1994) contains a single T7 RNA polymerase promoter followed by the cDNA sequence for the 70-, 14-, and 32-kDa subunits of hRPA. pET expression plasmid, pET-3a, was obtained from W. Studier and coworkers (Studier et al., 1990).

An N-terminal deletion of RPA70 missing residues 1−168 was generated and an expression vector similar to p11d-tRPA constructed. This plasmid, ptRPA·70Δ1−168, contains a single T7 RNA polymerase promoter, the coding sequence of RPA70 lacking residues 1−168 and the cDNA sequences for the 14- and 32-kDa subunits of hRPA. The construction of this mutant and its functional characterization will be described elsewhere (X. V. Gomes and M. S. Wold, manuscript submitted).

DNA manipulation. Restriction endonucleases and T4 DNA polymerase were used according to manufacturer's recommendations. Oligonucleotides were radiolabeled with [γ-^{32}P]ATP using polynucleotide kinase (Ausubel et al., 1989). Polymerase chain reactions (PCR) were performed with Vent DNA Polymerase (New England BioLabs) in a DNA Thermal Cycler (Perkin-Elmer). DNA amplification conditions were 25 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 3 min. PCR products and DNA fragments were isolated from 1% agarose gels run in 1× TAE using the Geneclean II kit (BIO 101, La Jolla, CA) according to manufacturer's specifications. Ligation reactions and transformations were as described (Ausubel et al., 1989). Recombinant plasmids were transformed into strain *E. coli* DH5α and isolated by the boiling lysis method (Ausubel et al., 1989). DNA sequencing was carried out using an Applied Biosystems 373A automatic DNA sequencer at the DNA Core Facility at the University of Iowa.

Construction of N-Terminal Deletion of the 32-kDa Subunit of hRPA. A mutagenic PCR primer to the coding sequence of RPA32 was synthesized; S33A-TS (5′-GCAC-C**TGCGCA**AGCCGAA-3′) contained nucleotide changes (underlined) to alter serine 33 to an alanine and to generate a *Fsp*I restriction site (bold). The mutagenic primer and a second primer, #35 [5′-ATGCTAGTTATTGCTCAGCGG-3′, corresponding to sequences downstream from the *Bam*HI site in all pET expression vectors (Studier et al., 1990)] were used to amplify a ∼0.9-kb fragment containing a 3′ region of the coding sequence of RPA32 from plasmid p3a-RPA32 (Henricksen et al., 1994). The 0.9-kb PCR fragment was isolated and ligated into pUC18 digested with *Sma*I to generate plasmid pUC-RPA32s33a3′. pUC-RPA32s33a3′ was digested with *Fsp*I and *Eco*RI generating a 0.9-kb restriction fragment. The fragment was isolated and ligated to pET-3a (Studier et al., 1990) digested with *Nhe*I, treated with T4 DNA polymerase to generate blunt ends and then digested with *Eco*RI. The subsequent vector, p3a-RPA32Δ1−

33, contains the coding sequence for RPA32 starting at glutamine 34. Sequence analysis confirmed that the fragment of RPA32 was maintained in the correct reading frame. p3a-RPA32Δ1−33 was digested with *Xba*I, treated with T4 DNA polymerase to generate blunt ends, and then digested with *Aat*II. The resulting 1-kb fragment was ligated to p11d-tRPA digested with *Sna*BI and *Aat*II. The subsequent expression vector, ptRPA·32Δ1−33, contains a single T7 RNA polymerase promoter followed by the coding sequence for RPA70, RPA14, and RPA32Δ1−33 with each preceded by a Shine−Dalgarno binding site. To place the deletion mutant in frame with an initiator methionine, three additional amino acids were fused at the N-terminus. Thus, rhRPA32Δ1−33 begins methionine-alanine-arginine and then continues with the coding sequence of hRPA32 starting at glutamine 34.

Induction and Purification of Wild-Type and Mutant rhRPA. p11d-tRPA, ptRPA·70Δ1−168, p11d-RPA70Δ442−616, p3d-RPA14/32, and ptRPA·32Δ1−33 were individually transformed into BL21(DE3), grown, and induced as described (Henricksen et al., 1994; Gomes & Wold, 1995). Recombinant hRPA (rhRPA), recombinant hRPA·70Δ1−168 (rhRPA·70N168), RPA70Δ442−616 (ΔC442), and rhRPA32·14 were purified as described (Henricksen et al., 1994; Gomes & Wold, 1995; X. V. Gomes and M. S. Wold, manuscript submitted). rhRPA·32Δ1−33 had chromatographic properties identical to wild-type RPA and was purified to apparent homogeneity (see Figure 3). The yield of rhRPA·32Δ1−33 varied between 0.5−1 mg of protein/L of induced culture.

Partial Proteolysis of rhRPA. The indicated amounts of rhRPA or mutant RPA complex were digested with either 25 ng of bromelain, 50 ng of trypsin, or 62 ng of chymotrypsin in 50 μL of HI buffer at 37 °C. At the times indicated, aliquots containing ∼1 μg of protein were removed, and proteolysis was terminated by boiling for 5 min in sample loading buffer. Even using this quenching procedure, the proteases took a finite amount of time to be inactivated. Thus, a low level of proteolysis was observed in zero minute time points. The proteolyzed products were separated on an 8−14% SDS−polyacrylamide gel (SDS−PAGE) (Laemmli, 1970) and analyzed either by staining with silver (Ausubel et al., 1989) or immunoblotting. All experimental results were confirmed in independent experiments. Variations in the rate of digestion were observed between individual experiments and with different protease preparations; however, the proteolytic fragments observed and their order of appearance were consistent throughout these studies.

Immunoblotting and Monoclonal Antibody Epitope Mapping of RPA70. Protein samples were separated on an 8−14% SDS−PAGE (Laemmli, 1970) and transferred to nitrocellulose (Bio-Rad) using a PolyBlot Electrotransfer System from Millipore as per manufacturer's specifications. RPA subunits and the various proteolytic fragments were detected using monoclonal antibodies to RPA70: αSSB70A (mab70A) (Kenny et al., 1990), αSSB70C (mab70C) (Kenny et al., 1990), RPA9 (mabRPA9; B. Stillman, CSHL), and 81 (mab81; Wold and Kelly, unpublished results); RPA32: 71 (Erdile et al., 1990) and polyclonal serum to RPA14: RPA3 (C. Umbricht and T. Kelly, Johns Hopkins). All of these antibodies are specific for a single subunit of hRPA, and no cross-subunit reactivity was observed in any experiment (data not shown). Secondary antibodies, sheep anti-mouse or goat anti-rabbit horseradish peroxidase conjugate (Sigma), were used to detect primary antibodies using an ECL chemiluminescense kit (Amersham) as recommended by manufacturer. Stripping and reprobing of nitrocellulose membranes were performed as recommended by the manufacturer.

Table 1: Recognition of RPA70 Mutants by Monoclonal Antibodies[a]

protein	monoclonal antibodies			
	mab70A	mab81	mab70C	mabRPA9
RPA70[b]	+	+	+	+
RPA70Δ507−616[c]	+	+	+	+
RPA70ΔC442−616[c]	+	+	+	+
RPA70Δ373−616[c]	+	+	+	−
RPA70Δ327−616[c]	+	+	+	−
RPA70Δ250−616[c]	+	+	−	−
RPA70Δ169−616[c]	+	+	−	−
RPA70Δ1−66[d]	nd	−	+	+
RPA70Δ1−112[d]	nd	−	+	+
RPA70Δ1−168[d]	nd	−	+	+
RPA70Δ1−236[d]	nd	−	−	+
RPA70Δ1−382[d]	nd	−	−	+

[a] Recognition of RPA70 mutants by individual monoclonal antibodies. Approximately 1−2 μg of cell lysate from induced cultures containing p11d-RPA70 (RPA70) or pET-11d containing individual RPA70 deletion mutants were separated on a 8−14% SDS−PAGE, transferred onto nitrocellulose, and probed with individual monoclonal antibodies to RPA70 as described in Experimental Procedures. (+) Recognized by indicated antibody on immunoblot; (−) not recognized by indicated antibody on immunoblot; (nd) not determined. [b] Wild-type RPA70. [c] C-terminal RPA mutants in which numbers refer to amino acids deleted (Gomes & Wold, 1995). [d] N-terminal RPA mutants in which numbers refer to amino acids deleted (X. V. Gomes and M. S. Wold, manuscript submitted).

The epitopes recognized by each monoclonal antibody to RPA70 were mapped using a series of six C-terminal and five N-terminal deletion mutants of RPA70 (Gomes & Wold, 1995; X. V. Gomes and M. S. Wold, manuscript submitted). Wild-type RPA70 and each deletion mutant were expressed individually in BL21(DE3) cells (Gomes & Wold, 1995). The resulting cell lysates were separated on an 8−14% SDS−PAGE, transferred to nitrocellulose and probed sequentially with individual monoclonal antibodies to RPA70. The results of this analysis are summarized in Table 1. These experiments identified the region of RPA70 recognized by each monoclonal antibody. These data indicated that mabRPA9 recognizes an epitope between residues 373 and 441, mab70A and mab81 recognize epitopes located between residues 1 and 168, and antibody mab70C recognizes an epitope between residues 168 and 326.

RESULTS

Proteolysis of RPA70. We examined the solution structure of rhRPA by analyzing its sensitivity to different proteases. Figure 1 shows the cleavage pattern of rhRPA at various times of digestion with either bromelain or trypsin. Bromelain is a nonspecific protease that probes the accessibility of the peptide bonds in proteins. Bromelain rapidly degraded RPA into distinct proteolytic fragments (Figure 1A, note polypeptides of 52, 33, and 16 kDa). Trypsin specifically cleaves peptide bonds on the carboxyl-terminal side of lysine and arginine residues providing a more limited/specific probe of protein conformation. The amino acid sequence of the three subunits of RPA predicts 71, 22, and 11 potential trypsin-cleavage sites in the 70-, 32-, and 14-kDa subunits

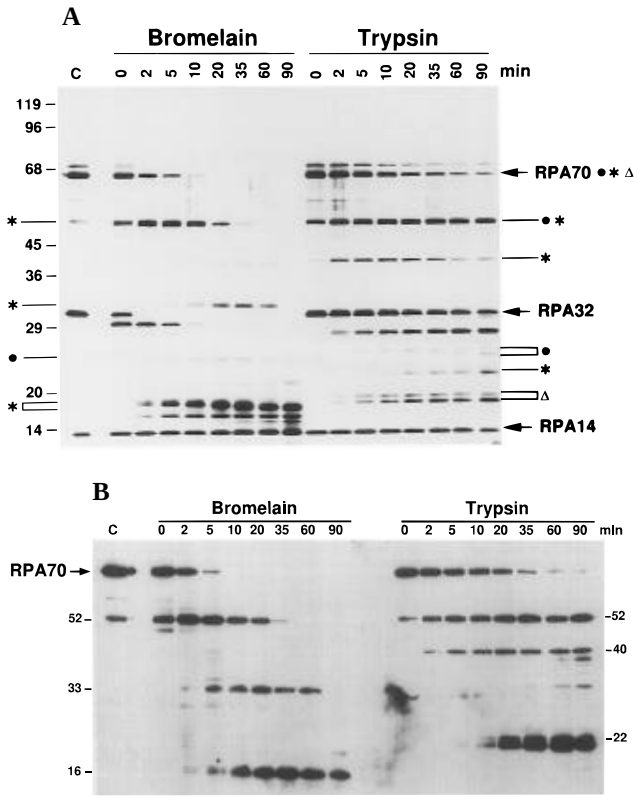

FIGURE 1: Partial proteolysis of rhRPA with bromelain and trypsin. Eight micrograms of rhRPA was digested with either trypsin or bromelain for the times indicated as described in Experimental Procedures. (A) Shown is an 8−14% SDS−PAGE gel stained with silver. The position of the molecular mass markers (dashes) and the position of the 70-, 32- and 14-kDa subunits of rhRPA (arrows) are indicated. Polypeptides recognized by various antibodies are also indicated: ●, mabRPA9; *, mab70C; △, mab81. Lane C contains purified rhRPA. (B) Immunoblot of proteolyzed rhRPA probed with mab70C. Position of full length RPA70 is indicated (arrow). Approximate molecular mass in kDa of proteolytic fragments is shown. Lane C contains purified rhRPA.

of RPA, respectively. However, digestion of rhRPA by trypsin resulted in the generation of a small number of distinct proteolytic fragments similar in size to those obtained after digestion by bromelain. Chymotrypsin specifically cleaves peptide bonds on the carboxyl-terminal side of aromatic amino acids. Digestion of rhRPA with chymotrypsin resulted in a cleavage pattern similar to the pattern of cleavage seen with trypsin (data not shown). These data indicate that, despite different specificities, the same regions of RPA were sensitive to digestion by all three proteases. Thus, each protease is probably recognizing the same structural elements of RPA.

Each lane in Figure 1A contains ~1 μg of rhRPA. At such high levels of RPA, a small amount of proteolyzed RPA70 is detected in the starting fraction (Figure 1A, lane C). In addition, a minor contaminant slightly larger than RPA70 is observed in this fraction of RPA. This protein did not react with antibodies to RPA and is unrelated to RPA (compare lane C in Figure1A to lane C in Figure 1B).

Using a series of deletion mutants of RPA70 (Gomes & Wold, 1995; X. V. Gomes and M. S. Wold, manuscript submitted), we mapped the regions of RPA70 recognized by individual monoclonal antibodies (Table 1). This information allowed us to identify the specific regions of RPA70 that were present in each of the proteolytic fragments. The polypeptides present after various times of digestion were separated by SDS−PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis. Figure 1B shows an immunoblot of rhRPA digested with either bromelain or trypsin and probed with monoclonal antibody mab70C. Only a subset of the proteolytic fragments of rhRPA observed after staining with silver cross-reacted with mab70C (compare Figure 1 panels A and B). Digestion of rhRPA by bromelain initially resulted in a ~52-kDa proteolytic fragment. Polypeptides of ~33 and ~16 kDa were detected by mab70C at later time points. After digestion of rhRPA with trypsin, polypeptides of ~52, 40, and 22 kDa were detected by mab70C. Similar analysis was performed with other monoclonal antibodies to RPA70 (data not shown). The specific proteolytic fragments of RPA70 recognized by mab70C and other monoclonal antibodies are summarized in Figure 1A. The initial ~52-kDa cleavage product of RPA70, observed with all proteases, cross-reacted with monoclonal antibodies mabRPA9 and mab70C but not with mab81. Since antibody mab81 recognizes the N-terminal region of RPA70, the ~52-kDa fragment must arise from cleavage events near the N-terminus. Thus, the initial cleavage event of RPA70 by either bromelain, trypsin, or chymotrypsin removes ~18 kDa from the N-terminus. After this initial cleavage event, the C-terminal ~52-kDa fragment of RPA70 is sensitive to subsequent digestion into smaller fragments. The number and size of these secondary cleavage products differed for each protease used (Figure 1A, see also Discussion).

Proteolysis of the 32- and 14-kDa Subunits of RPA. These studies also provided information about the susceptibility of the 32- and 14-kDa subunits of RPA to proteolysis. After treatment with either bromelain or trypsin, the initial digestion product of RPA32 was a ~28−30-kDa polypeptide (Figure 1A). Immunoblot analysis confirmed that these polypeptides were cleavage products of RPA32 (Figure 2). This truncated polypeptide was relatively resistant to further cleavage by trypsin but was rapidly degraded by bromelain. A slightly larger (31-kDa) initial cleavage product was obtained when rhRPA was digested with chymotrypsin (data not shown). Examination of the amino acid sequence of RPA32 indicated that the polypeptides obtained with trypsin and chymotrypsin were most consistent with the initial cleavage of RPA32 being near the N-terminus (data not shown). To confirm this hypothesis, a mutant RPA complex lacking amino acid residues 1−33 of RPA32 (rhRPA·32Δ1−33) was digested with trypsin. The 32-kDa subunit of both rhRPA·32Δ1−33 and wild-type rhRPA is cleaved rapidly to a ~28-kDa proteolytic fragment (32′, Figure 3). Since digestion of rhRPA·32Δ1−33 and wild-type rhRPA yielded the same ~28-kDa polypeptide, the initial cleavage event of RPA32 by trypsin must remove the N-terminus. Antibodies to RPA32 were unable to detect any other cleavage products suggesting that either there were no other stable proteolytic fragments or that subsequent cleavage events removed the antigenic determinants.

The 32- and 14-kDa subunits of RPA can form a stable complex (rhRPA32·14) in the absence of the 70-kDa subunit of RPA (Henricksen et al., 1994; Stigger et al., 1994). Therefore, we also examined the protease sensitivity of RPA32 in the rhRPA32·14 complex to determine if there were differences between the structure of rhRPA32·14 and the native RPA complex. The proteolytic fragments obtained after digestion of rhRPA32·14 with trypsin were identical

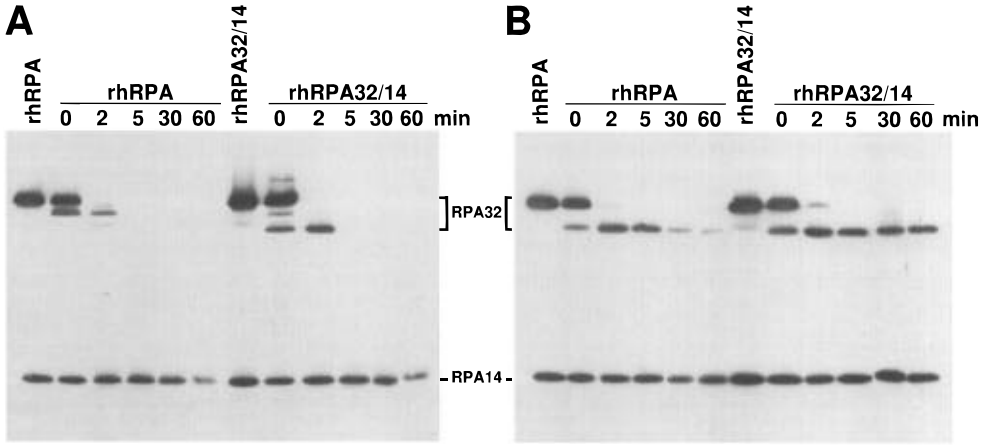

FIGURE 2: Immunoblot of proteolysis of the 32·14 complex. rhRPA (7.5 μg, ~70 pmol) or 4 μg of rhRPA32/14 (~70 pmol) was digested as described in Experimental Procedures with either (A) bromelain (25 ng) or (B) trypsin (25 ng) for the times indicated. The immunoblot was probed with anti-RPA32 monoclonal antibody 71 (Erdile et al., 1990) and anti-RPA14 polyclonal antibody RPA3 (Umbricht et al., 1993) to visualize both subunits. Positions of RPA14 and full-length and proteolytic fragments of RPA32 are indicated. The left-most lane of each set contains the purified protein fraction (vertical labels).

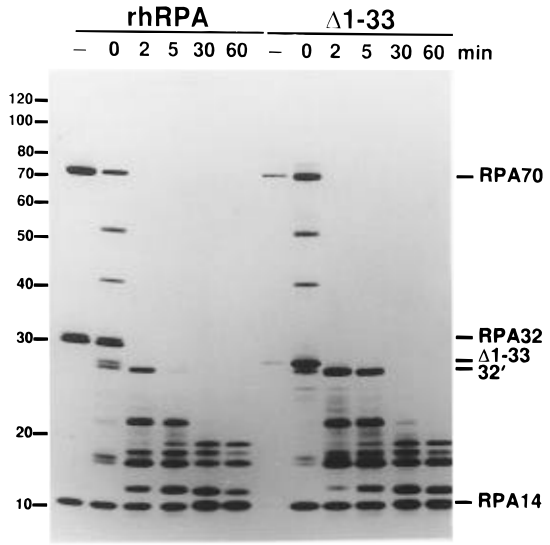

FIGURE 3: Digestion of rhRPA·32Δ1−33 with trypsin. Approximately 7 μg of either rhRPA or rhRPA·32Δ1−33 were treated with 5 ng of trypsin. At the times indicated, aliquots containing ~1 μg (10 pmol) were removed and proteolysis terminated by boiling for 5 min in sample loading buffer. An 8−14% SDS−PAGE stained with silver is shown. The position of the subunits of RPA are indicated including the ~28-kDa proteolytic fragment of RPA32 (32′). The position of the molecular mass markers is shown on the left in kDa.

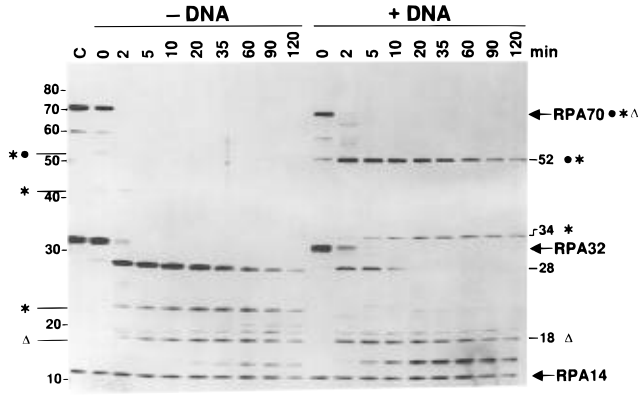

FIGURE 4: Digestion of rhRPA with trypsin in the absence (−DNA) or presence (+DNA) of ssDNA. Eight micrograms of rhRPA was digested with 50 ng of trypsin. Where indicated 138 fmol of DNA (oligo(dT)$_{30}$) was added prior to the addition of trypsin. Digestion products were separated on an 8−14% SDS−PAGE and stained with silver. Lane C contains purified rhRPA. The position of the molecular mass markers is shown on the left in kDa. The arrows indicate the position of the 70-, 32-, and 14-kDa subunits of RPA. Approximate molecular mass in kDa of proteolytic fragments is shown.

to those obtained with the heterotrimeric RPA complex (Figure 2); RPA32 was degraded rapidly into a ~28-kDa polypeptide which was resistant to further digestion. With bromelain, the initial cleavage product of the rhRPA32·14 complex was slightly smaller than observed with rhRPA: ~28-kDa rather than a ~30-kDa polypeptide (see Figure 2A). Similar results were also obtained when the proteolytic fragments of rhRPA32·14 were visualized by staining with silver (data not shown). Thus, the proteolytic sensitivity of the 32-kDa subunit in the 32·14 subcomplex is similar to the entire RPA complex. These results indicate that the structure of RPA32 in rhRPA32·14 is similar to its structure in the heterotrimeric RPA complex. The sensitivity of the N-terminal residues of RPA32 to proteolytic cleavage in both complexes indicates that the N-terminus of RPA32 is either on the surface of the complex and/or in an extended conformation.

In contrast to both RPA70 and RPA32, RPA14 seemed unaffected with any protease tested (Figures 1A and 2). There are 11 potential trypsin cleavage sites and 10 potential chymotrypsin sites present in this 121-residue subunit. RPA14 was resistant to cleavage both in the native RPA complex and in the rhRPA32·14 subcomplex. Even after extensive digestion in which RPA70 and RPA32 were degraded, RPA14 remained resistant to proteolytic digestion (Figure1A, 60 and 90 min digestion with bromelain). These results suggest that the intrinsic conformation of RPA14 is highly resistant to protease cleavage.

Changes Observed in Protease Sensitivity of rhRPA in the Presence of ssDNA. We next examined whether interactions with ssDNA changed the sensitivity of RPA to protease digestion. rhRPA was incubated with a 2-fold molar excess of oligo(dT)$_{30}$ prior to digestion with protease. Previous analysis of RPA binding has demonstrated that under these conditions only one molecule of rhRPA binds to oligo(dT)$_{30}$ (Kim et al., 1994; Kim & Wold, 1995). Figure 4 shows the cleavage of rhRPA by trypsin in the absence or presence of oligo(dT)$_{30}$. In the experiment shown, a preparation of

trypsin was used that gave a more rapid rate of proteolysis than that in the experiment shown in Figure 1. These conditions emphasize differences in protease sensitivity of RPA in the presence of ssDNA. In this assay, in the absence of ssDNA, the initial ~52-kDa fragment of RPA70 was degraded in less than 2 min of digestion (compare -DNA at 0 and 2 min, Figure 4). In contrast, in the presence of ssDNA, RPA70 was degraded rapidly to the ~52-kDa fragment; however, this ~52-kDa fragment was now resistant to secondary cleavage events. In the presence of ssDNA, the ~52-kDa fragment was slowly digested to a ~34-kDa fragment (Figure 4). This is in contrast to digestion in the absence of ssDNA in which a ~40-kDa secondary cleavage fragment was observed (compare Figures 1 and 4). In similar experiments with bromelain, the ~52-kDa fragment was also more resistant to cleavage in the presence of ssDNA (data not shown). Similar changes in proteolytic digestion pattern were also observed when rhRPA was digested in the presence of poly(dT) with a average length of 3000 nt (data not shown). Immunoblot analysis of rhRPA digested in the presence of oligo$(dT)_{30}$ using monoclonal antibodies to RPA70 indicated that with the exception of the ~34-kDa trypsin fragment, the proteolytic fragments produced in the presence of ssDNA were identical to those observed in the absence of ssDNA (Figure 4; see also Figure 1). We conclude that binding of RPA to ssDNA causes a reduced rate of proteolytic cleavage of RPA70 and resulted in the detection of at least one additional trypsin cleavage site. These changes could either be caused by the bound ssDNA sterically blocking access of proteases or by a DNA-induced conformational change in RPA.

Binding of the rhRPA complex to ssDNA also altered both the pattern and rate of cleavage of RPA32. In the absence of ssDNA, RPA32 was proteolyzed rapidly into a ~28-kDa fragment and was then resistant to further proteolysis (Figure 4, see also Figure 1). In the presence of DNA, RPA32 became more sensitive to trypsin and the ~28-kDa fragment was rapidly degraded (Figure 4). In addition, the site of the initial proteolytic cleavage of the N-terminus of RPA32 changed in the presence of ssDNA. In experiments containing less trypsin, a novel ~31-kDa fragment of RPA32 was observed in the present of ssDNA (data not shown; see also Figure 4, 2 min digestion). Thus, binding to ssDNA caused RPA32 to become more sensitive to proteolytic cleavage and resulted in the detection of at least one additional cleavage site. Currently, there is no evidence that RPA32 directly interacts with ssDNA; therefore, we conclude that the observed changes in the proteolytic sensitivity of RPA32 are the result of DNA-induced conformational changes in RPA32 and/or the RPA complex.

DNA-Binding Activity of Proteolytic Fragments of RPA. The 70-kDa subunit of RPA contains intrinsic DNA-binding activity (Wold et al., 1989), and the ssDNA-binding site in RPA70 has been mapped to the region contained in residues 1−442 (Gomes & Wold, 1995). Proteolysis of RPA initially causes the cleavage of approximately 170 residues from the N-terminus of RPA70 (see above); therefore, we examined whether these residues were important for ssDNA binding activity. The ability of specific proteolytic fragments to interact with ssDNA were determined by southwestern analysis (Figure 5). rhRPA was preincubated with oligo-$(dT)_{30}$ and then digested with trypsin. The proteolytic fragments were separated on an 8−14% SDS−PAGE,

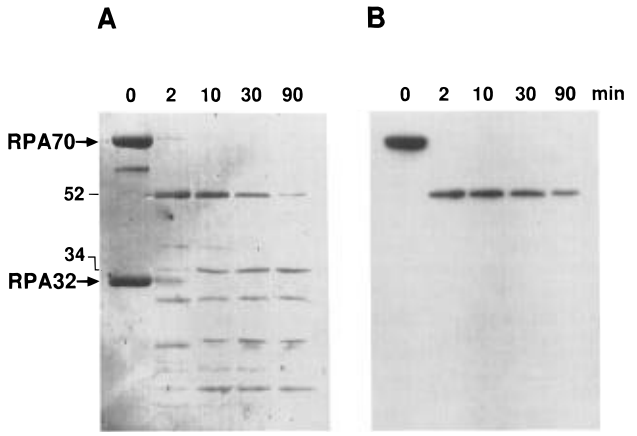

FIGURE 5: Southwestern analysis of rhRPA proteolyzed with trypsin. Southwestern analysis was performed as described (Wold et al., 1989). Approximately 100 μg of rhRPA was incubated with 1 nmol of oligo$(dT)_{30}$ prior to digestion with 200 ng of trypsin in 100 μL of HI buffer at 37 °C. At indicated times, aliquots containing ~10 μg of protein were removed and proteolysis terminated by boiling for 5 min in sample loading buffer. The proteolyzed products were separated on an 8−14% SDS−PAGE and transferred to nitrocellulose as described for an immunoblot. The nitrocellulose was blocked by incubating in phosphate buffered saline (150 mM sodium chloride, 10 mM sodium phosphate, pH 7.05, 1 mM EDTA, 1 mM sodium azide) containing 4% (w/v) bovine serum albumin and 0.2% (v/v) Triton X-100 for 30 min at 25 °C. The nitrocellulose was then incubated with ~10 pmol (1×10^7 cpm) ^{32}P-labeled oligo-$(dT)_{30}$ at 25 °C for 60 min, washed three times with phosphate-buffered saline containing 0.2% Triton X-100 and analyzed by autoradiography. Panel A shows the nitrocellulose blot stained with India ink. Panel B is an autoradiogram of the same nitrocellulose blot probed with ^{32}P-labeled oligo$(dT)_{30}$. The arrows indicate the position of the full length RPA70, RPA32, and the 52-kDa proteolytic fragment.

transferred onto a nitrocellulose membrane, and probed with radioactively labeled oligo$(dT)_{30}$. To accurately monitor the rate of digestion and efficiency of protein transfer, the nitrocellulose membrane was stained with India ink (Figure 5A). Only full length RPA70 and the initial ~52-kDa proteolytic fragment bound ssDNA (Figure 5B). All other proteolytic fragments had no ssDNA-binding activity in these assays even though they were present in amounts similar to the ~52-kDa fragment (compare band intensities of the ~52-kDa fragment at 90 min to intensities of smaller proteolytic fragments). Identical results were obtained after digestion of RPA in the absence of ssDNA except that digestion proceeded much more rapidly (data not shown). These results indicate that the N-terminal ~170 residues are not essential for DNA-binding activity.

Proteolysis of RPA70 Deletion Mutants. The high sensitivity of the N-terminus of RPA70 proteases and the finding that these residues are not essential for ssDNA binding activity suggest that the ~170 residues of RPA70 may be a separate structural domain. A deletion mutant of RPA70 was made in which residues 1−168 were deleted, rhRPA·70N168. This ~52-kDa mutant forms a stable complex with RPA32 and RPA14 and supports SV40 DNA replication (X. V. Gomes and M. S. Wold, manuscript submitted). If the N-terminus of RPA70 is a separate structural domain, this mutant would be expected to have proteolytic properties identical to those of the wild-type RPA complex. When rhRPA·70N168 was digested with trypsin in absence of ssDNA, the population of proteolytic fragments was identical to those seen with full length RPA70 (compare -DNA in

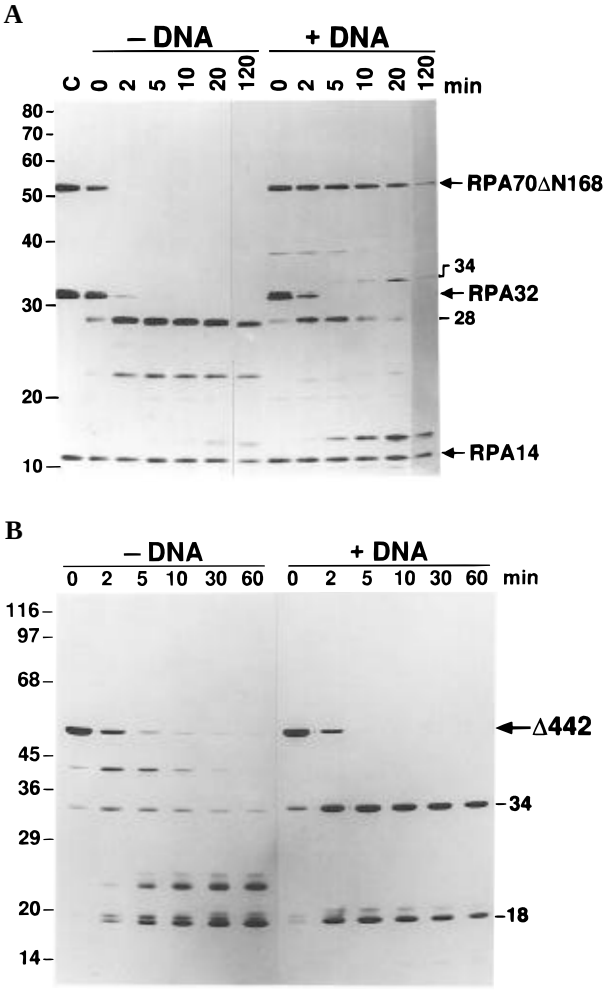

FIGURE 6: Digestion of RPA deletion mutants in the presence or absence of ssDNA. Nine micrograms of rhRPA·70ΔN168 (A) or 12 μg of ΔC442 (Δ442) (B) were digested with either 50 or 12.5 ng, respectively, of trypsin in the absence (−DNA) or presence (+DNA) of 168 fmol of DNA (oligo(dT)$_{30}$). At the times indicated, aliquots of protein were removed, and proteolysis was terminated by boiling for 5 min in sample loading buffer. The proteolyzed products were separated on an 8−14% SDS−PAGE and analyzed by staining with silver. Lanes C contain either undigested rhRPA·70ΔN168 (A) or ΔC442 (B). The position of the molecular mass markers is shown on the left (in kDa). The position of the mutated RPA70 subunits (arrows) and the molecular mass in kDa of the major proteolytic fragments observed are indicated on the right.

Figure 6A with Figure 4). (As expected, the ∼18-kDa N-terminal fragment of wild-type RPA70 was not observed when rhRPA·70ΔN168 was digested.) In the presence of ssDNA, the deleted RPA70 subunit became much more resistant to digestion by trypsin and RPA32 became more sensitive to trypsin digestion (Figure 6A). No new sites of protease cleavage were detected on rhRPA·70ΔN168. These data are consistent with this mutant having a similar structure to the wild-type RPA complex and with the N-terminus of RPA70 being a distinct structural domain.

These studies suggest that RPA70 is composed of multiple structural domains. If the DNA-binding site of RPA70 is part of a distinct structural domain, then mutations that disrupt other regions of RPA70 would be predicted to have little effect on the structure of this domain. Previously, we have characterized a C-terminal deletion mutant of RPA70, RPA70Δ442−616 (ΔC442), that binds to oligonucleotides with an affinity close to that of wild-type RPA (Gomes & Wold, 1995). Hydrodynamic studies indicated that the 50-kDa ΔC442 maintains a distinct structure in solution in the absence of the 32- and 14-kDa subunits of RPA (Gomes & Wold, 1995). We subjected ΔC442 to limited proteolysis in the presence and absence of ssDNA to determine if the solution structure of ΔC442 differed significantly from that of rhRPA. The cleavage products obtained with ΔC442 were similar to those obtained with full-length rhRPA (Figure 6B). (Since 32- and 14-kDa subunits were absent, polypeptides derived from these subunits were not observed.) As with rhRPA, the initial cleavage event removed ∼18-kDa from the N-terminus of ΔC442 (Figure 6B). In the absence of ssDNA, the resulting ∼34-kDa C-terminal fragment was cleaved into a smaller ∼24-kDa fragment (Figure 6B) while in the presence of ssDNA, additional cleavage of the 34-kDa fragment was not observed (Figure 6B). This DNA-dependent protection of ΔC442 is identical to that observed with RPA70 in the complete RPA complex. This strongly suggests that the structure of the first 441 residues of RPA70 is similar in ΔC442 and wild-type RPA and is consistent with RPA70 being composed of distinct structural domains. These results suggest that interactions with the 32- and 14-kDa subunits do not affect the structure of this region of 70-kDa subunit of RPA.

DISCUSSION

hRPA is a very stable, heterotrimeric complex in solution. The heterotrimeric structure of hRPA and related homologues is unique among single-stranded DNA-binding proteins. Currently, little is known about the structure of hRPA complex. In this study, we performed limited proteolysis with trypsin, chymotrypsin, or bromelain to examine the solution structure of rhRPA. Both wild-type and mutant forms of rhRPA were digested with a protease for various intervals of time, separated on SDS−PAGE, and characterized by staining and immunoblot analysis. The sites of cleavage on each subunit of RPA identified are summarized in Figure 7. Only a limited number of the possible cleavage sites were found to be accessible in fully-folded rhRPA. These sites are likely to indicate exposed regions of the protein such as surface loops or interdomain regions. These studies allow us to draw general conclusions about the structure of the individual subunits of hRPA and the hRPA complex.

We found that RPA14 was highly resistant to protease digestion under all conditions used in these studies. This could indicate that RPA14 is buried when part of the RPA complex or the 32·14 subcomplex. However, we observed that RPA14 was resistant to digestion even after complete digestion of the 70- and 32-kDa subunits (Figure 1). We conclude that the 14-kDa subunit of RPA intrinsically has a protease resistant structure. Studies with antibodies to RPA14 indicate that the N- and C-terminus of RPA14 are not accessible in heterotrimeric RPA (Umbricht et al., 1993; Stigger et al., 1994); however, because polyclonal serum to RPA14 specifically inhibits replication, there must be regions of RPA14 accessible to solution in the RPA complex (Umbricht et al., 1993).

RPA32 was partially resistant to protease digestion. All proteases tested rapidly removed the N-terminus of RPA32 (Figures 1 and 2). The precise cleavage site varied between proteases (summarized in Figure 7), but in all cases the initial cleavage events removed approximately 30−40 residues from

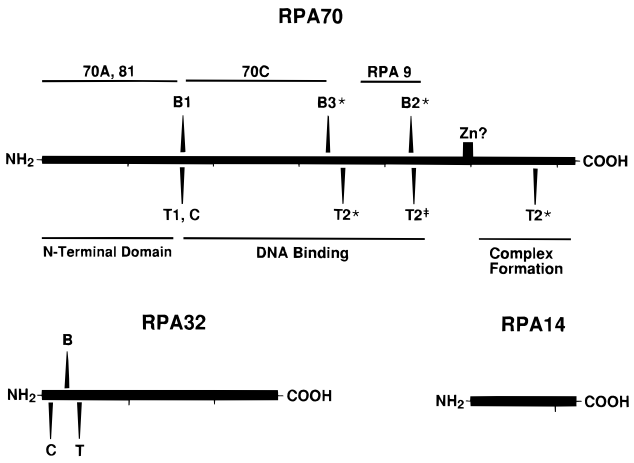

FIGURE 7: Preliminary map of the functional domains of RPA subunits. The figure shows schematic of RPA70, RPA32, and RPA14. Ticks are positioned every 100 amino acids. Triangles indicate protease sensitive sites mapped in these studies with numbers indicating order of cleavage: T, trypsin; B, bromelain; C, chymotrypsin. (*) Sites of cleavage which are protected in the presence of ssDNA. (‡) Site of cleavage observed in the presence of ssDNA. The position of the conserved, putative zinc finger motif in RPA70 is indicated by solid box (Zn?). Lines under the schematic of RPA70 indicate the location of putative domains (see text for details). Lines above the schematic of RPA70 indicate the regions recognized by individual monoclonal antibodies.

the N-terminus. The rapid rate of cleavage and the variation in the precise sites of cleavage suggest that the N-terminus of RPA32 is in an exposed configuration. After proteolytic cleavage of the N-terminus, the 28-kDa C-terminal fragment of RPA32 was resistant to further digestion by trypsin and chymotrypsin but sensitive to bromelain. Similar results were obtained with both the native RPA complex and a subcomplex of the 32- and 14-kDa subunits of RPA suggesting that the structure of RPA32 is similar in both complexes.

Phosphorylation of RPA32 is strongly stimulated by RPA binding to ssDNA (Fotedar & Roberts, 1992; Henricksen et al., 1994). Recently, it has been shown that the N-terminus of RPA32 is important for phosphorylation of RPA but not required for replication activity (Lee & Kim, 1995; L. A. Henricksen, T. Carter, A. Dutta, and M. S. Wold, manuscript submitted). We have shown that all phosphorylation occurs on the N-terminal domain of RPA32 (L. A. Henricksen, T. Carter, A. Dutta, and M. S. Wold, manuscript submitted). The studies presented here provide a structural context for these findings. We hypothesize that RPA32 is composed of two structural domains: a small exposed N-terminal domain required for phosphorylation and a C-terminal domain required for subunit interactions and activity. The observed changes in the protease sensitivity indicate that RPA32 changes conformation when the RPA complex binds to ssDNA. These changes are probably responsible for the increased level of phosphorylation observed in the presence of ssDNA. These properties are consistent with the possibility that the N-terminus of RPA32 is a regulatory domain. Lee and Kim (1995) have recently shown that the C-terminus of RPA32 is essential for replication activity. Our studies provide no evidence for the C-terminus of RPA32 being a third structural domain. Thus, it is possible that the C-terminus of RPA32 may have both a structural and a functional role. Recently, several mutations in the gene encoding RPA32 have been characterized in yeast (Santocanale et al., 1995). These mutations cause defects in DNA replication and repair and are all located in the large C-terminal domain of RPA32.

The digestion pattern of RPA70 was complex. In order to identify the sites of proteolytic cleavage on RPA70, we used monoclonal antibodies to identify the various proteolytic fragments of RPA70. We mapped the regions of RPA70 recognized by different monoclonal antibodies using a series of C-terminal deletion mutants of RPA70 (Table 1). This information allowed us to localize the cleavage sites on RPA70. With all proteases tested the initial cleavage event on RPA70 was ~170 residues from the N-terminus generating ~18- and ~52-kDa fragments (Figure 7). After the initial cleavage event, the ~18-kDa N-terminal fragment was partially resistant to further digestion by trypsin (Figure 1A) or chymotrypsin (data not shown). In contrast, the ~52-kDa C-terminal fragment was digested into smaller fragments by all proteases tested. The specific sites of cleavage of the ~52-kDa fragment varied with each protease used (Figure 7), suggesting that after removal of the N-terminus, much of the C-terminus of RPA70 is accessible to proteases.

We found that there were significant changes in the digestion pattern of RPA70 after RPA bound ssDNA. In the presence of ssDNA, the initial proteolytic event which generated the ~52-kDa C-terminal fragment was not affected. However, this fragment was more resistant to digestion by trypsin (Figure 4), bromelain, or chymotrypsin (data not shown). The sites protected in the presence of ssDNA are located primarily but not exclusively in the DNA-binding domain (Figure 7). A similar DNA-induced protection was also seen in the case of two deletion mutants of RPA70: rhRPA·ΔN168 and ΔC442 (see Figure 6). Binding of rhRPA to ssDNA also altered the proteolytic digestion pattern of RPA32; in the presence of ssDNA, RPA32 had increased sensitivity to proteases and an additional cleavage site was observed. These changes in RPA70 and RPA32 could be due to DNA protecting protease sensitive sites or by a DNA-induced conformational change. We hypothesize that both processes are occurring. Most of the sites that become resistant to digestion in the presence of ssDNA are located in the DNA-binding domain (Figure 7). These sites are likely to be protected from cleavage by steric effects of ssDNA binding. However, steric effects are unlikely to explain the increase sensitivity of RPA32 in the presence of ssDNA. In addition, sites not located in the DNA-binding domain were also protected from cleavage. These sites include one trypsin cleavage site near the C-terminus of RPA70 and one in RPA32. These data are most consistent with there being a conformational change in RPA upon ssDNA binding. As described above, this conformational change probably makes the N-terminus of RPA32 more accessible to protein kinases (Fotedar & Roberts, 1992; Henricksen et al., 1994).

RPA70 has intrinsic DNA-binding activity. We determined whether any of the proteolytic fragments of RPA70 retained this activity by southwestern analysis. Only the full length RPA70 and the ~52-kDa proteolytic fragment bound DNA. This suggests that the N-terminal ~18-kDa fragment is not required for the interactions of RPA70 with DNA and that all other cleavage events disrupt the DNA-binding domain of RPA70. Previously, we have mapped the location of the DNA-binding domain of RPA70 to the N-terminus between residues 1−442 (Gomes & Wold, 1995). The

southwestern data shown in Figure 5 suggest that residues 1−170 are dispensable for DNA-binding activity thereby localizing the DNA-binding domain to residues 170−442.

The C-terminal region of RPA70 between residues 507 and 616 is required for the formation of a stable heterotrimeric complex with RPA32 and RPA14 (Gomes & Wold, 1995; X. V. Gomes and M. S. Wold, manuscript submitted). We conclude that there are at least three functional domains in RPA70: an ∼18-kDa (∼170 amino acid) N-terminal domain that is not required for interactions with DNA, a central domain that interacts directly with ssDNA, and a C-terminal domain required for complex formation. Proteolytic analysis of ΔC442 showed that deletion of the C-terminus of RPA70 did not change the proteolytic sensitivity of the first 441 amino acids. This suggests that ΔC442 has a similar conformation to that of full-length RPA70. This would be consistent with the central and C-terminal domains being in distinct structural domains; however, additional experimentation will be needed to demonstrate this.

Functional studies that examined the effect of specific antibodies to hRPA *in vitro* provide additional information about the possible function of the domains of RPA70. Mab70A specifically inhibits RPA stimulation of DNA polymerase α while mab70C specifically inhibits RPA stimulation of both T antigen-dependent DNA-unwinding and DNA polymerase *δ* activity (Kenny et al., 1990). We show above that mab70A interacts with the N-terminal domain of RPA70 and mab70C interacts with the central domain. Thus, it is likely that the N-terminal domain of RPA70 is involved with interactions with DNA polymerase α and the central domain of RPA70 may be involved in interactions with DNA polymerase *δ*. Because the central region of RPA70 also interacts with ssDNA, it is also possible that the inhibition by mab70C may be an indirect effect of antibody binding.

There is a high level of conservation between the RPA homologues in yeast and humans; therefore, it is likely that the structural domains described above are conserved in yeast RPA. Recently, genetic studies have identified mutations in the yeast homologue of RPA70 that disrupt RPA function *in vivo* (Longhese et al., 1994; Firmenich et al., 1995; Smith & Rothstein, 1995). Several mutations with severe defects are located in the N-terminal domain of RPA70; these include a point mutation at G77 (G77D) (Firmenich et al., 1995) and two amino acid insertions between residues S7 and R8 (T1) and Y96 and V97 (M2) (Longhese et al., 1994). The phenotypes of these mutations include reduced rates of DNA repair and recombination (G77D), lethality (T1), and temperature sensitive growth coupled to reduced rates of replication and repair (M2). These results indicate that the N-terminal domain of RPA is important for RPA function *in vivo* and may be involved in multiple cellular processes. Two other mutants, M4 [a two amino acid insertion between T211 and W212 (Longhese et al., 1994)] and a point mutation at residue D228, rfa1-D228Y (Smith & Rothstein, 1995), are located in the central DNA-binding domain. These mutants also have slow-growth and repair-deficient phenotypes and provide evidence that the central domain is essential for RPA function *in vivo*.

The changes in protease sensitivity observed with RPA are similar to those observed previously with prokaryotic single-stranded DNA-binding proteins. In the case of *E. coli* single-stranded DNA-binding protein, binding to ssDNA increased the sensitivity to proteases but only if the DNA being bound was long enough to allow cooperative binding (Williams et al., 1983). When T4 gene 32 protein was treated with proteases in the presence of ssDNA, one region of the protein was protected and a second region of the protein became more sensitive (Hosoda & Moise, 1978; Williams & Konigsberg, 1978). Again these changes were only observed with DNA fragments long enough to allow cooperative binding (Hosoda & Moise, 1978; Williams & Konigsberg, 1978). We find that RPA70 become more resistant to protease digestion after binding to ssDNA while RPA32 becomes more sensitive. In contrast to the prokaryotic single-stranded DNA-binding proteins, both short and long oligonucleotides had similar effects on the proteolysis of RPA (data not shown). Thus, these studies suggest that cooperative binding of RPA is not necessary for DNA-induced changes in protease sensitivity of RPA. This could reflect the fact that unlike both *E. coli* single-stranded DNA-binding protein and T4 gene 32 protein, RPA binds ssDNA with low cooperativity (Kim et al., 1994; Kim & Wold, 1995). Additional experiments are needed to examine the effects of cooperative binding on RPA conformation.

It has been hypothesized that the formation of the RPA complex requires an ordered assembly of subunits with the 32- and 14-kDa subunits interacting to form a stable subcomplex that then interacts with the 70-kDa subunit to form a complete RPA complex (Henricksen et al., 1994). The studies presented here are consistent with this model. The protease sensitivity of RPA14 and RPA32 is similar in both the 32•14 subcomplex and in the native RPA heterotrimer. In addition, the 70-kDa subunit of RPA is very sensitive to protease digestion, consistent with this subunit being on the exterior of the heterotrimeric RPA complex. Additional structural studies will be needed to test this model and define the structure of RPA more precisely.

ACKNOWLEDGMENT

We thank Madeline A. Shea for scientific discussions and suggestions during these studies. We thank B. Stillman and J. Hurwitz for monoclonal antibodies to RPA and C. Umbricht and T. Kelly for polyclonal serum. We thank the members of the Wold laboratory for scientific discussions and critical reading of the manuscript. We thank the University of Iowa DNA Core Facility for oligonucleotide synthesis. These studies were supported by Public Health Service Grant GM44721 from the General Medicine Institute.

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BI9526995