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Cloning of the Large Subunit of Replication Protein A (RPA) from Yeast *Saccharomyces cerevisiae* and Its DNA Binding Activity through Redox Potential

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Eukaryotic replication protein A (RPA) is a single-stranded(ss) DNA binding protein with multiple functions in DNA replication, repair, and genetic recombination. The 70-kDa subunit of eukaryotic RPA contains a conserved four cysteine-type zinc-finger motif that has been implicated in the regulation of DNA replication and repair. Recently, we described a novel function for the zinc-finger motif in the regulation of human RPA's ssDNA binding activity through reduction-oxidation (redox). Here, we show that yeast RPA's ssDNA binding activity is regulated by redox potential through its RPA32 and/or RPA14 subunits. Yeast RPA requires a reducing agent, such as dithiothreitol (DTT), for its ssDNA binding activity. Also, under non-reducing conditions, its DNA binding activity decreases 20 fold. In contrast, the RPA70 subunit does not require DTT for its DNA binding activity and is not affected by the redox condition. These results suggest that all three subunits are required for the regulation of RPA's DNA binding activity through redox potential.

Keywords: Replication protein A (RPA), RPA70, Single-stranded DNA binding protein, Redox

Introduction

Replication Protein-A (RPA) is a three-subunit single-stranded DNA binding protein (SSB) that was originally identified as a human protein that is required for SV40 DNA replication in vitro (Wobbe *et al*., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). RPA binds with high affinity to single-stranded DNA (ssDNA), and with much lower affinity to double-stranded DNA (dsDNA) and RNA. Although several eukaryotic SSBs were previously identified from both viruses and cells, RPA was the first cellular SSB that was shown to be directly involved in DNA metabolism (Wold, 1997). Human RPA (hRPA) is a stable complex of three subunits of 70, 32, and 14-kDa. Homologous heterotrimeric ssDNA-binding proteins have been identified in all of the eukaryotes that were examined. This indicates that hRPA is a member of a family of eukaryotic SSBs (Wold, 1997). Yeast *Saccharomyces cerevisiae* RPA (scRPA) is composed of 69-(RPA1; encoded by RFA1), 36-(RPA2; encoded by RFA2), and 13-kDa (RPA3; encoded by RFA3) subunits. Each subunit is essential for viability. The large subunit of RPA will be referred to as RPA70, the intermediate subunit as RPA32, and the small subunit as RPA14 (Wold, 1997; Sibenaller *et al*., 1998). RPA also functions in DNA repair and recombination (Wold, 1997).

The 70-kDa subunit of eukaryotic RPA (RPA70) contains a conserved four cysteine-type zinc-finger motif that has been implicated in the regulation of DNA replication and repair (Carty *el al* 1994; Park *et al*., 1999b). Unlike other zinc-finger proteins, the RPA zinc-finger motif is not a DNA-binding component. Recently, we described a novel function of the zinc-finger motif in the regulation of human RPA's ssDNA binding activity through redox potential (Park *et al*., 1999b; You *et al*., 2000).

In this study, we found that all three subunits are required for the redox regulation of yeast RPA's ssDNA binding activity.

Materials and Methods

Strains and media The yeast strains that were used in this study are DBY1830 (*a/α ade2/ + lys2-801/ + his3-Δ200// + his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-1/trp1-1*) and DBY1829

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(α lys2-801 his3-Δ200 ura3-52 trp1-1). Cells were routinely grown at 30°C in a synthetic minimal medium SD with necessary supplements (Sherman *et al*., 1974). *Escherichia coli* strain DH5α was used for the propagation of plasmids. Transferring cells from glucose to a galactose-containing medium caused the GAL1 promoter to become derepressed and transcription to be induced.

Amplification of yeast RPA70 by polymerase chain reaction The reaction mixture for the PCR contained 130 ng/μl of template DNA (DBY1829 chromosomal DNA), 10 pmol of each primer, 12.5 mM dNTP, 10× buffer (100 mM Tris-HCI (pH 8.3), 500 mM KCl, 25 mM $MgCl_2$, 0.01% gelatin), and 5 units of Taq polymerase.

The oligonucleotide primers were designed as follows: forward primer 5'-AAGCTTCCAGATTATACTTACAAGA-3', reverse primer 5'-GGATCCTTGATTATTTGATACATTA-3'. The forward primer was designed to contain *Hin*dIII restriction sites (underlined). The reverse primer contained *Bam*HI restriction sites (underlined).

A thermal cycler was set to the following cycle parameters: 5 min at 94°C (1 cycles), 1 min at 95°C, 1 min at 50°C, 3 min at 72°C (30 cycles), 7 min at 72°C (1 cycle), and rapid cooling to 4°C.

TOPO cloning reaction and *E. coli* transformation The reaction mixture contained fresh PCR product 3 μl, salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) 1 μl, TOPO vector 1 μl (Invitrogen, San Diego, USA), in a total volume of 10 μl and was incubated at room temperature for 30 min. The PCR product was ligated into pYES2.1/V5 His-TOPO, and the recombinant vector transformed into competent *E. coli*.

Yeast transformation and RPA70 induction The constructed RPA70 expression vector was transformed into yeast strain DBY1830. A single colony that contained the pYES2.l/V5 His-TOPO construct was inoculated into 15 ml of a SD medium that contained 2% glucose and necessary supplements. The cultures were grown overnight at 30°C with shaking. After centrifugation at 1500 × *g* for 5 min at 4°C, the cell pellet was resuspended in 30 ml of an induction medium and continued to grow at 30°C with shaking. The induction medium contained 2% galactose instead of glucose.

The cells were harvested by centrifuging at 1500 × *g* for 5 min at 4°C, and washed once with sterile water, then centrifuged for 30 sec at top speed in the microcentifuge. The cell pellet was washed again in 500 μl of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, I mM PMSF), and centrifuged at 1500 × g for 5 min at 4°C. The cells were resuspended in a volume of breaking buffer to obtain an OD600 of 100. An equal volume of acid-washed glass beads was added. The mixture was vortexed for 30 sec followed by 30 sec on ice. This procedure was repeated four times for a total of 4 min to lyse the cells. The lysed samples that contained glass beads were centrifuged in a microcentrifuge for 10 min at maximum speed. The lysate (supernatant) was transferred to a fresh microcentrifuge tube and a 5× SDS-PAGE sample buffer (60 mM Tris-HCI, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) was added to a final concentration of 1×.

Purification of RPA and RPA70 The RPA and RPA70 were purified according to the procedure described previously (Park *et al*., 1999b). Briefly, the lysates from the cultures of yeast 1830 cells were adjusted to 0.5 M NaCl/and filtered through a 0.45 μm filter. They were then loaded onto an Affi-Gel Blue column (2.5 × 8 cm), equilibrated with buffer A (25 mM Tris-HCI, pH 7.5, 10% glycerol, 1 mM DTT, 0.5 mM EDTA, 0.02% NP-40, 0.1 mM PMSF, 0.2 μg/ml antipain, 0.l μg/ml leupeptin) that contained 0.5 M NaCl. The column was successively washed with 20 column volumes of buffer A that contained 0.5 M NaCl and 0.8 M NaCl. The proteins were eluted with buffer A that contained 2.5 m NaCl plus 40%(v/v) ethyleneglycol. The protein-containing fractions were diluted with buffer A to 0.5 M NaCl, and applied to a ssDNA-cellulose column (10 mg of protein/ml bed volume of ssDNA-cellulose), equilibrated with buffer A that contained 0.5 M NaCl. After washing the column with buffer A that contained 0.5 M NaCl and 0.8 M NaCl, proteins were eluted with buffer A that contained 1.5 M NaCl plus 50%(v/v) ethyleneglycol. Peak protein fractions from the 1.5 M NaCl ethyleneglycol wash were pooled and dialyzed against buffer A that contained 200 mM NaCl and 20% glucose (Park *et al*., 1999b). The RPA or RPA70 was analyzed using Western blotting as well as 12% SDS-PAGE.

ssDNA binding assay $Oligo(dT)_{50}$ was 5'-end labeled with [γ-^{32}P]ATP (Du Pont, Wilnington, USA) and T4 polynucleotide kinase (USB) based on the manufacturer's instructions. The 20 ng of RPA or RPA70 was incubated with 100 fmol of 5'-^{32}P-labeled $oligo(dT)_{50}$ at room temperature for 15 min in the reaction mixtures (30 μl) that contained 50 mM HEPES-KOH (pH 7.8), 10 mM $MgCl_2$, polydI : dC (0.2 μg), BSA (0.2 μg/μl), and indicated amounts (Fig. 2 and Fig. 3) of DTT or NaCl. Protein-DNA complexes were analyzed using 5% polyacrylamide gels in 1× TBE buffer (acrylamide: bisacrylamide = 79 : 1). The gels were dried and exposed to x-ray films (Kodak, Rochester, USA). The bands of interest were excised from the gels and measured for radioactivity using a Beckman Scintillation Counter LS 6500 (Beckman, Jersey, USA).

Results and Discussion

The PCR amplification of a DNA fragment that encoded the yeast RPA70 was conducted as described in Materials and Methods. The amplification of the 1.8 kb DNA fragment was verified by agarose gel electrophoresis.

To examine whether the ssDNA binding activity of wild-type yeast RPA and 70-kDa subunit RPA(RPA70) are affected by the redox condition *in vitro*, we purified them. The RPA was purified from yeast DBY1830 cells. For the purification of the RPA complex, we used two kinds of the column chromatography method. The lysates from yeast DBY1830 cells were loaded onto an Affi-Gel Blue column and the fractions that were eluted from the Affi-Gel Blue column were loaded onto a single-stranded DNA-cellulose column. We could see that the purified RPA showed homogeneity (Fig. 1A, lane 2). RPA70 was purified from yeast DBY1830 cells that contained the RPA70-TOPO gene. We used the same method as the RPA purification. We could see the purified RPA70 (Fig. 1A, lane 3). RPA and RPA70 were confirmed by a Western blot analysis (Fig. 1B).

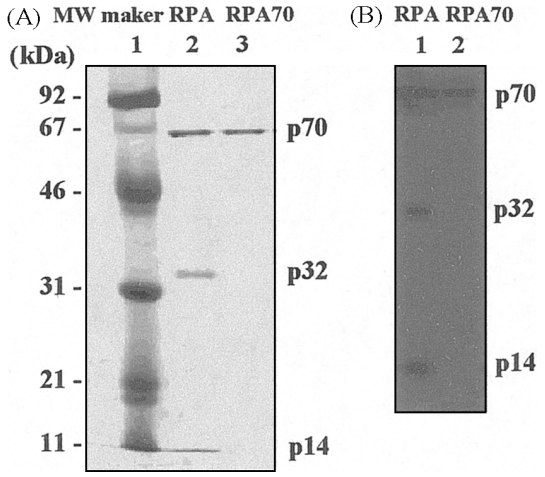

Fig. 1. SDS-Polyacrylamide gel electrophoresis (panel A) and Western blotting (panel B) of purified RPA and RPA70. Proteins were electrophoretically separated on a 12% SDS-PAGE and visualized by Coomassie Blue staining. Lane 1 in panel A is protein marker. Western blot was performed using anti-yeast RPA polyclonal antibodies as a probe.

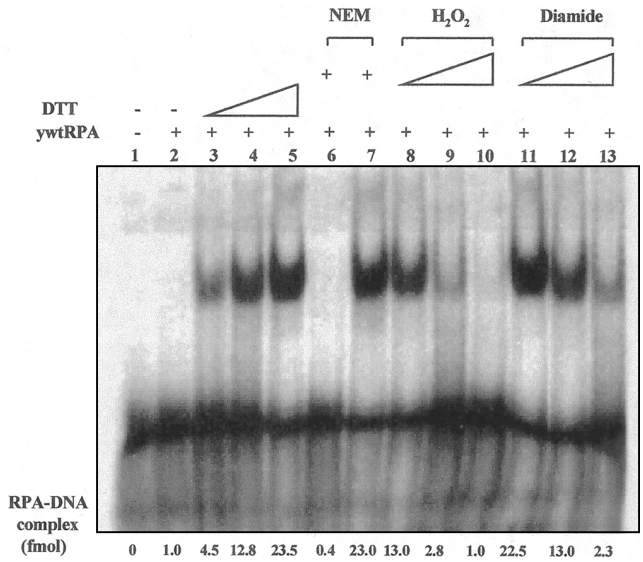

Fig. 2. Effect of DTT, H_2O_2, diamide, and NEM on yRPA's ssDNA binding activity. Wild type RPA (20 ng) was preincubated with 0 mM (lane 2), 0.5 mM (lane 3), 2.5 mM (lane 4), and 12.5 mM DTT (lane 5). Then 100 fmol of 5'-^{32}P-labeled oligo $(dT)_{50}$ was added and allowed to incubate for 15 min at room temperature. No RPA was included in lane 1. RPA (20 ng) was preincubated with 12.5 mM DTT and increasing amounts of H_2O_2 (0.3 mM, 0.75 mM, and 1.5 mM H_2O_2 in lanes 8-10, respectively). RPA (20 ng) was preincubated with 12.5 mM DTT and increasing amounts of diamide (0.3 mM, 0.75 mM, and 1.5 mM diamide in lanes 11-13, respectively). RPA (20 ng) was preincubated with 9 mM NEM and increasing amounts of DTT (0 mM, and 12.5 mM DTT in lanes 6-7, respectively). The RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis (acrylamide: bisacrylamide = 79 : 1). For quantitation, regions of the RPA-DNA complex that are shown in the figure were excised and measured for radioactivity.

In order to understand the regulatory function of RPA, we examined whether the RPA ssDNA binding activity is affected by redox potential. For this, RPA was preincubated with various amounts of DTT and examined for its interaction with oligo $(dT)_{50}$ in the presence of 200 mM NaCl. The RPA-DNA complex was analyzed by an electrophoretic mobility shift assay on polyacrylamide gel under equilibrium conditions (Fig. 2, lanes 2-5). A very low RPA-DNA complex was formed in the absence of DTT, which was stimulated up to 20-fold by the addition of DTT (Fig. 2, lanes 2-5). To examine the redox regulation further, RPA was treated with the oxidizing agent diamide and H_2O_2 in the presence of 12.5 mM DTT. The addition of increasing amounts of H_2O_2 (Fig. 2, lanes 8-10) and diamide (Fig. 2, lanes 11-13) gradually decreased the formation of the RPA-DNA complex. These results strongly suggest that RPA ssDNA binding activity is regulated by redox potential.

Cellular redox plays a key role in the modulating DNA binding activity of several transcription factors, such as Fos-Jun (Abate *et al*., 1990), CCAAT-binding factor (CBF; also known as NF-Y) (Nakshatri *et al*., 1996), p53 (Jayaraman *et al*., 1997), and Pax 8 (Tell *et al*., 1998). A mutational analysis of these proteins indicated that cysteine residues are involved in redox-regulation (Xanthoudakis and Curran, 1992; Nakshatri *et al*., 1996; Tell *et al*., 1998). RPA's ssDNA binding activity was sensitive to H_2O_2 and diamide (Fig. 2, lanes 8-13), a chemical that catalyzes the oxidation of free sulfhydryl groups (Kosower and Kosower, 1987). This suggests a possible involvement of cysteine residues in the redox-dependent DNA binding activity of RPA. We therefore examined whether RPA's ssDNA binding activity is sensitive to N-ethylmaleimide (NEM), which alkylates the free sulfhydryl group (Fig. 2, lanes 6-7). NEM treatment completely abolished RPA's ssDNA binding activity (Fig. 2, lane 6). RPA's DNA binding activity was unaffected by the NEM treatment after the addition of DTT, which suggests that the effect of NEM on RPA's DNA binding activity is due to the alkylation of the sulfhydryl group (Fig. 2, lane 7). The inhibitory effect of NEM on RPA's ssDNA binding activity strongly indicates that cysteine residues are involved in the redox regulation of RPA's DNA binding activity.

We also examined whether RPA32 and/or RPA14 subunits are involved in the redox regulation of its ssDNA binding activity. In contrast to RPA, RPA70 formed a stable complex with ssDNA, even under non-reducing conditions. The addition of DTT had no effect on its DNA binding activity (Fig. 3, lanes 2-5). To further examine the redox regulation, RPA70 was treated with the oxidizing agent, H_2O_2, in the presence of 0.4 mM DTT (Fig. 3, lanes 6-8). The addition of increasing amounts of H_2O_2 significantly reduced the RPA-ssDNA complex, whereas RPA70 was much less affected by

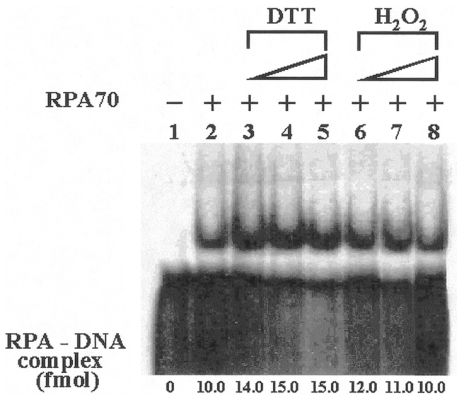

Fig. 3. Effect of DTT and H_2O_2 on RPA70's ssDNA binding activity. RPA70 (20 ng) was preincubated with 0 mM (lane 2), 0.5 mM (lane 3), 2.5 mM (lane 4), and 12.5 mM DTT (lane 5), and then 100 fmol of 5'-^{32}P-labeled oligo $(dT)_{50}$ was added and allowed to incubate for 15 min at room temperature. No RPA70 was included in lane 1. RPA70 (20 ng) was preincubated with 12.5 mM DTT and increasing amounts of H_2O_2 (0.3 mM, 0.75 mM, and 1.5 mM H_2O_2 in lanes 6-8, respectively). The RPA70-DNA complex was analyzed by a 5% polycarylamide gel electrophoresis (acrylamide: bisacrylamide = 79 : 1).

the H_2O_2 treatment. These results strongly suggest that both the RRA32 and/or RPA 14 are involved in the redox regulation of RPA's ssDNA binding activity.

In a previous study, we found that RPA's ssDNA binding activity is regulated by redox through the cysteines in a putative zinc finger domain (Park *el al*., 1999b). Zinc fingers are autonomous folding units and highly versatile structural elements that are found in many sequence-specific DNA binding proteins (Clarke and Berg, 1998). Specific cysteine residues have been identified in several non-zinc finger transcription factors that are involved in the redox regulation of their DNA binding activity (Abate *et al*., 1990; Nakshatri *el al*., 1996; Jayaraman *el al*., 1997; Tell *et al*., 1998). In RPA, cysteine residues are not only involved in the redox regulation of RPA's DNA binding activity, but also the key component of its zinc-finger structure (Park *et al*., 1999b). A number of DNA-binding proteins have been identified. Their DNA binding activity is regulated by redox, although the role of the zinc finger in regulation is unclear (Hutchison *et al*., 1991; Wu *el al*., 1996; Gowen *et al*., 1998).

In this study, we found that RPA's ssDNA binding activity is regulated by redox potential through RPA32 and/or RPA14. The clectrophoretic mobility shift assays (described here for yeast RPA) clearly demonstrated that binding to the corresponding DNA targets is extremely sensitive to redox conditions, whereas RPA70 is not.

We therefore hypothesize that two small subunits (RPA32 and RPA14), as well as the zinc finger domain in RPA70, are key regulatory elements in modulating RPA's DNA binding activity. The 4-Cys zinc fingers are highly conserved among eukaryotic RPA. The 4-Cys zinc-finger contains Zn(II) that coordinates four cysteine residues tetrahedrally (Luisi *et al*., 1991). Under reducing conditions, the zinc finger structure is favorably formed and Zn(II), buried in the interior, stabilizes the module by binding 4 cysteines. Under nonreducing conditions, however, oxidation of the Zn(II)-thiolate bond induces the releases of Zn(II) from the zinc finger, which promotes the formation of a disulfide bond between cysteines in the zinc finger domain and other cysteines (either inside or outside of the zinc finger domain) (Casadevall *el al*., 1998). The formation of the disulfide bond may induce structural changes, which interfere with the DNA binding domain of RPA70; or alternatively, the formation of a disulfide bond may change the protein conformation, which affects the DNA binding activity of this protein.

In this way, RPA's DNA binding activity by redox is controlled by the zinc-finger domain of p70 in a two-small subunits dependent manner. These studies provide an interesting insight into the structure and function relationship of a multiprotein complex, so that the role of a specific domain (or one subunit) is regulated by the other subunit. Further structural analysis is necessary to evaluate the detailed conformational change.

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