Replication Protein A (RPA) Regulates Its DNA Binding Activity through Redox

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Replication protein A (RPA; also known as a single-strand DNA (ssDNA) binding protein with multiple functions in DNA replication, repair, and genetic recombination) is composed of three subunits (70-, 34- and 11-kDa; p70, p34, and p11, respectively),[1-4] which tightly associate with each other in various eukaryotic RPA preparations, suggesting its functional activity is highly conserved. The large subunit (p70) is responsible for its ssDNA binding activity.[1,5] Human RPA shares a high degree of amino acid identity (41%) with the p70 subunit of yeast RPA. The high degree of homology (81%) shared by these proteins, between species as distantly related as yeast and humans, implies that the fundamental mechanisms of DNA replication are likely to be very well conserved throughout the eukaryotic kingdom.

Recent studies suggested that RPA function is regulated in response to DNA damage[6] and its ssDNA binding activity may be involved in this regulatory event.[7] In previous studies, we showed that human RPAs ssDNA binding activity was regulated by redox potential through its zinc finger domain.[8]

In an effort to understand RPA's regulatory function, we examined whether yeast RPAs ssDNA binding activity is affected by redox. Yeast RPA[9] was treated with increasing amounts of DTT and examined for its interaction with oligo $(dT)_{50}$ in the presence of 200 mM NaCl. RPA-DNA complex was analyzed by electrophoretic mobility shift assays[10] which separates the complex from free DNA (Figure 1a). Very poor RPA-DNA complex was formed in the absence of DTT (Figure 1a; lane 2), which was stimulated by the addition of DTT up to 10-fold (Figure 1a; lanes 3-6). This result suggests that redox affects yeast RPA's DNA binding affinity such that it is significantly enhanced under reducing conditions. To examine redox regulation further, yeast RPA was treated with the oxidizing agent, H_2O_2 in the presence of 0.4 mM DTT. Addition of increasing amount of H_2O_2 gradually decreased the formation of RPA-DNA complex (Figure 1a; lanes 6-10), compared with that under reducing condition (Figure 1a; lane 6). These results strongly suggest that yeast RPAs ssDNA binding activity is also regulated by redox potential.

Cellular redox plays a key role in modulating DNA binding activity of several transcription factors, such as Fos-Jun,[11] CCAAT-binding factor (CBF; also known as NF-Y),[12] p53,[13] and Pax 8.[14] Mutational analysis of these proteins indicated that cysteine residues are involved in redox regulation.[12,14,15]

We also examined whether the cysteine residue of human RPA's DNA binding domain is involved in redox regulation of its ssDNA binding activity. In contrast to yeast wild-type

(ywt) RPA, human mutant C289A (hmC289A), (cys-to-ala mutation at amino acids 289),[9,16] formed a stable complex with ssDNA even under non-reducing conditions. The addition of DTT had no effect on its DNA binding activity (Figure 1b; lanes 2-6). To further examine redox regulation, human mutant (hmC289A) RPA were treated with the oxidizing agent, H_2O_2, in the presence of 0.4 mM DTT (Figure 1b). The addition of increasing amounts of H_2O_2 significantly reduced ywt RPA-ssDNA complex, whereas hmC289A RPA was much less affected by H_2O_2 treatment (Figure 1b; lanes 7-10). These results strongly suggest that the cysteine in DNA binding domain is involved in the redox regulation of RPA's ssDNA binding activity and that the cysteine residues of p70, in particular cysteine 289, are essential for this regulation.

In previous study, we found that RPA's ssDNA binding activity is regulated by redox through the cysteine 486 in a putative zinc finger domain.[8] Zinc fingers are autonomous folding units and highly versatile structural elements found in many sequence-specific DNA binding proteins.[17] Specific cysteine residues have been identified in several non-zinc finger transcription factors to be involved in redox regulation of their DNA binding activity.[11-14] In RPA, cysteine residues are not only involved in redox regulation of RPA's DNA binding activity, but also the key component of its zinc-finger structure.[8] A number of DNA-binding proteins have been identified in which their
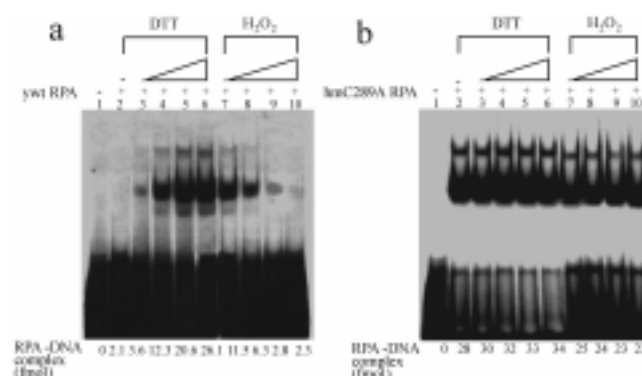

Figure 1. Effects of reducing and oxidizing agents on ssDNA binding activity. **a.** Yeast wild-type (ywt) RPA's DNA binding activity is stimulated by reducing agent, DTT, but is inhibited by the oxidizing agent, H_2O_2. **b.** Cysteine 289 is involved in redox regulation of RPA's DNA binding activity. Effect of DTT on ssDNA binding activity of human mutant (hmC289A) RPA. RPA (20 ng) was preincubated with 0 mM (lane 2), 0.02 mM (lane 3), 0.2 mM (lane 4), 2 mM (lane 5), and 20 mM DTT (lane 6) and then 100 fmol of 5'-^{32}P-labeled oligo(dT)$_{50}$ was added and allow to incubate for 15 min at room temperature. No RPA was included in lane 1. For quantitation, regions of RPA-DNA complex shown in the figure were excised and measured for radioactivity. RPA (20 ng) was preincubated with 1 mM DTT and increasing amounts of H_2O_2 (0.2, 0.5, 1.0, and 2.0 mM H_2O_2 in lanes 7-10, respectively).

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a

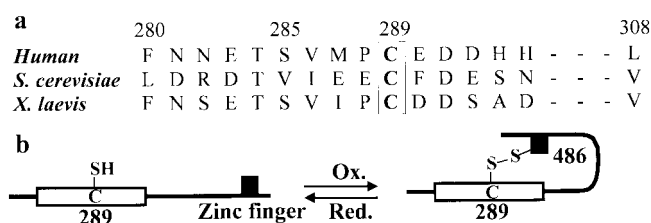|	280					285				289					308
Human	F	N	N	E	T	S	V	M	P	**C**	E	D	D	H	H	- - - L
S. cerevisiae	L	D	R	D	T	V	I	E	E	**C**	F	D	E	S	N	- - - V
X. laevis	F	N	S	E	T	S	V	I	P	**C**	D	D	S	A	D	- - - V

b

Figure 2. A proposed model for redox-mediated change the structure and regulation of RPAs DNA binding activity. **a.** Conserved cysteine in DNA binding domain is indicated in bold-type. **b.** A stable structure is formed under reducing condition. Under oxidizing condition, one disulfide bond is formed between cysteines 289 and 486. Open bar indicates DNA binding domain. *S. cerevisiae, Saccharoymces cerevisiae; X. laevis, Xenopus laevis.*

DNA binding activity is regulated by redox, although the role of the zinc finger in regulation is not clear.[18-20] In this study, we found that RPA's ssDNA binding activity is regulated by redox through the cysteine 289 in a DNA binding domain. The electrophoretic mobility shift assays, here described for yeast RPA, clearly demonstrated that binding to the corresponding DNA targets is extremely sensitive to redox conditions, whereas human mutant RPA (hmC289A) could not.

We therefore hypothesize that cysteine 289 and zinc finger domain in p70 are a key regulatory elements in modulating RPA's DNA binding activity. The cysteine 289 and 4-Cys zinc finger are highly conserved among eukaryotic RPA (Figure 2a). The 4-Cys zinc-finger contains Zn(II) which tetrahedrally coordinates four cysteine residues.[21] 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 Zn(II)-thiolate bond induces the releases of Zn(II) from the zinc finger, which promotes the formation of disulfide bond between the cysteine 289 and cysteine 486 (within of the zinc finger) (Figure 2b).[22] The formation of disulfide bond may induce a structural changes which interferes with the DNA binding domain of p70 (Figure 2b), or alternatively, the formation of disulfide bond may change the protein conformation which affects the DNA binding activity of this subunit.

The cells realize efficient production of ATP mainly through the glucose oxidation. However, this event leads to the generation of reactive oxygen species (ROS) such as H_2O_2, O_2^-, and $\cdot$OH radicals, that are by-products in electron transport processes into the mitochondria. To protect themselves from the harmful effect of oxygen, cells use primarily two defense mechanism, the enzymatic systems of catalase and superoxide dismutase, and the glutathione and TRX systems, which contribute to create an intracellular reducing environment. Therefore, through the induction of antioxidant systems, ROS constitute a useful tuning device for signal transduction. The redox environment may have profound effect on the structure and stability of DNA binding proteins. DNA-binding proteins are the major targets for redox regulation since they all contain cysteine residues that are essential for DNA binding activity.[17] Thus we conclude from this study that cysteins 289 and 486 in p70 of RPA have a unique role to regulate its DNA binding activity through redox change, probably function in response to various environmental stress.

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9. Yeast wild-type RPA and human mutant RPA were purified according to the Reference 2 and 8, respectively.
10. RPA-ssDNA binding assay: Oligo (dT)$_{50}$ was 5'-end labeled with [γ-^{32}P]ATP (Du Pont) and T4 polynucleotide kinase (USB) based on the manufacturer's instructions. The indicated amount of wild-type or mutant RPA was incubated with 100 fmol of 5'-^{32}P-labeled Oligo (dT)$_{50}$ at room temperature for 15 min in the reaction mixtures (30 mL) containing 50 mM Hepes-KOH (pH 7.8), 10 mM MgCl$_2$, polydI : dC (0.2 mg), BSA (0.2 μg/μL), and indicated amounts of DTT or NaCl. Protein-DNA complexes were analyzed using 5% polyacrylamide gels in 1x TBE buffer (acrylamide: bisacrylamide = 79 : 1). The gels were dried and exposed to x-ray films (Kodak). The bands of interest were excised from the gels and measured for radioactivity using a Beckman Scintillation Counter LS 6500.
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16. Plasmid constructs and site-directed mutagenesis: cDNA encoding the p70 subunit ofhuman RPA was cloned into baculovirus transfer vector pVL941-SW. For the change Cys-289' Ala, the plasmid pVL941SW-70 (carring wild-type p70 gene) and the fellowing primers were used in the PCR reaction, with the underlined nucleotides changes from the wild-type sequence: 5-TCC GTC ATG CCC <u>GCC</u> GAG GAC GAC CAT-3 and 5-ATG GTC GTC CTC <u>GGC</u> GGG CAT GAC GGA-3. The mutation procedure was performed according to the Stratagene protocol.
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