Zinc Finger of Replication Protein A, a Non-DNA Binding Element, Regulates Its DNA Binding Activity through Redox*

(Rceived for publication, March 3, 1999, and in revised form, July 20, 1999)

Jang-Su Park, Mu Wang, Su-Jung Park, and Suk-Hee Lee‡

From the Department of Biochemistry and Molecular Biology, the Indiana University Cancer Center and Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202

Eukaryotic replication protein A (RPA) is a single-stranded DNA-binding protein with multiple functions in DNA replication, repair, and genetic recombination. RPA contains an evolutionarily conserved 4-cysteine-type zinc finger motif (X_CX2_5CX3_2CX_C) that has a potential role in regulation of DNA replication and repair (Dong, J., Park, J.-S., and Lee, S.-H. (1999) Biochem. J. 337, 311–317 and Lin, Y.-L., Shivji, M. K. K., Chen, C., Kolodner, R., Wood, R. D., and Dutta, A. (1998) J. Biol. Chem. 273, 1453–1461), even though the zinc finger itself is not essential for its DNA binding activity (Kim, D. K., Stigter, E., and Lee, S.-H. (1996) J. Biol. Chem. 271, 15124–15129). Here, we show that RPA single-stranded DNA (ssDNA) binding activity is regulated by reduction-oxidation (redox) through its zinc finger domain. RPA-ssDNA interaction was stimulated 10-fold by the reducing agent, dithiothreitol (DTT), whereas treatment of RPA with oxidizing agent, diazene dicarboxylic acid bis[N,N-dimethylamide] (diamide), significantly reduced this interaction. The effect of diamide was reversed by the addition of excess DTT, suggesting that RPA ssDNA binding activity is regulated by redox. Redox regulation of RPA-ssDNA interaction was more effective in the presence of 0.2 mM NaCl or higher. Cellular redox factor, thioredoxin, was able to replace DTT in stimulation of RPA DNA binding activity, suggesting that redox protein may be involved in RPA modulation in vivo. In contrast to wild-type RPA, zinc finger mutant (cysteine to alanine mutation at amino acid 486) did not require DTT for its ssDNA binding activity and is not affected by redox. Together, these results suggest a novel function for a putative zinc finger in the regulation of RPA DNA binding activity through cellular redox.

The replication protein A (RPA); also known as human single-stranded DNA-binding protein) is a three-subunit complex (70–34 kDa; p70, p34, and p11, respectively) essential for DNA replication, nucleotide excision repair, and genetic recombination (54). In simian virus 40 (SV40) replication, RPA mediates unwinding of replication origin in the presence of SV40 T-antigen and topoisomerase I/II. During replication, it interacts with SV40 T-antigen and DNA polymerase α-primase (pol α-primase) complex (6, 7), which is necessary for the initiation of SV40 DNA replication (7–9). RPA is also involved in the elongation phase of DNA replication, because it stimulates pol α, pol δ, and pol ε activity on a primed template DNA (10, 11).

In nucleotide excision repair, RPA interacts with several key repair proteins, Xeroderma pigmentosum (XP) group A-complementing protein, XPA (12–15), XPG (13), and XPF-excision repair cross-complementation group 1 (16). RPA stabilizes the XPA-damaged DNA complex through the interaction with XPA, which appears to be essential for DNA repair (14, 17). RPA itself can interact with UV-damaged DNA (18); however, the physiological relevance of RPA-damaged DNA interaction in DNA repair is not clear. RPA is also involved in the later stage of nucleotide excision repair, gap-filling reaction, in collaboration with proliferating cell nuclear antigen, replication factor-C, and pol δ (or pol ε) (19). In homologous recombination, RPA physically interacts with Rad51 and Rad52, which appears to be essential for initiation of recombination (20–24).

The large subunit of RPA, p70, has multiple functional domains, including pol α stimulation, ssDNA binding, and a conserved zinc finger domain with 4-cysteine type (3, 5, 25). The ssDNA binding domain of RPA resides in the middle of p70 (3–5), and the structural analysis revealed that this domain consists of two homologous subdomains in tandem position (26). The DNA binding domain but not the polymerase stimulation domain is essential for the function of RPA in replication (27). Both yeast RPA and human RPA share a highly conserved putative metal binding domain of the 4-cysteine type (X_2_CX_5_4CX_2_1_CX_3_C) toward the C terminus (amino acids 478–503) (28, 29) of p70. Zinc finger domain in several DNA-binding proteins such as SP1 transcription factors and adenovirus DNA-binding protein plays a key role in the interaction with DNA (30–32). Deletion analysis indicated that the zinc finger domain of RPA, unlike others, is not essential for its ssDNA binding activity (2, 4, 27). However, mutation at the zinc finger domain differentially affected its function in replication and nucleotide excision repair (1, 2), suggesting a possible role for zinc finger domain in regulation.

In mammalian cells, DNA damage or heat shock induces inhibition of replication that can be efficiently reversed by the addition of human RPA (33, 34), suggesting a role for RPA in regulation of DNA replication in response to environmental stress. It is not clear how RPA is involved in stress-induced replication arrest; however, an observation with severe combined immunodeficient mice cells (35) argues that RPA phosphorylation by ionizing radiation correlates with its reduced ssDNA binding activity, suggesting a possible role of RPA.
phosphorylation in stress-induced replication arrest. In contrast, an in vitro experiment with hyperphosphorylated form of RPA demonstrated that DNA replication and repair activities were not affected by RPA p34 phosphorylation (36).

In this study, we found that RPA ssDNA binding activity is regulated through its zinc finger domain via reduction-oxidation (redox), one of the major regulatory mechanisms in response to environmental stress. A possible role for zinc finger domain in redox regulation is discussed below.

**EXPERIMENTAL PROCEDURES**

**Proteins and Chemicals**—Thioredoxin, N-ethylmaleimide (NEM), and diamide (diazene dicarboxylic acid bis[(N,N-dimethylamido)] were obtained from Sigma, and DTT was purchased from Roche Molecular Biochemicals.

**Preparation of Wild-type and Mutant RPA**—RPA was prepared according to the procedure described previously (37) with slight modifications. Briefly, lysates were prepared from insect cells (SF-9) coinfected with recombinant baculoviruses encoding wild-type p11, wild-type p34, and either wild-type or mutant p70. After adjusting the salt concentration to 0.5 M NaCl, lysates were loaded onto a ssDNA cellulose column equilibrated with buffer A (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.02% Nonidet P-40, 1 mM DTT, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, and 0.2 μg/ml antipain) containing 0.5 M NaCl. The column was successively washed with 20 column volumes of the buffer A containing 0.5 M NaCl and 0.8 M NaCl. The proteins were eluted with buffer A containing 2.0 M NaCl, 40% ethylene glycol. The eluted fractions were diluted 5-fold with buffer A and loaded onto an Affi-Gel Blue (Bio-Rad) column that was equilibrated with buffer A containing 0.5 M NaCl. After washing the column with buffer A containing 0.5 M NaCl and 0.8 M NaCl, proteins were eluted with buffer containing 2.5 M NaCl, 40% ethylene glycol. The RPA-containing fractions were pooled and dialyzed against buffer A containing 50 mM NaCl and further purified on a Q-Sepharose column with a linear salt gradient (50 mM to 0.4 M NaCl). All purification procedures were carried out at 4 °C, and during purification, RPA was monitored by immunoblotting using anti-p70 and -p34 antibodies (37). The fractions with at least 90% purity by Coomassie staining were collected and stored at −80 °C until use.

**RPA ssDNA Binding Assay**—Oligo(dT)$_{50}$ (ICN) and T4 polynucleotide kinase (Amersham Pharmacia Biochemicals) based on the manufacturer’s instructions. The indicated amount of wild-type or mutant RPA was incubated with 100 fmol of 5'-32P-labeled oligo(dT)$_{50}$ at room temperature for 15 min in the reaction mixtures (30 μl) containing 50 mM Hepes-KOH (pH 7.8), 10 mM MgCl$_2$, poly(dC)-poly(dC) (0.2 μg), bovine serum albumin (0.2 μg/μl), and indicated amounts of DTT or NaCl. Protein-DNA complexes were analyzed using 5% polyacrylamide gels in 1× Tris borate EDTA (acrylamide:bisacrylamide = 79:1). The gels were dried and exposed to x-ray films (Eastman Kodak Co). The bands of interest were excised from the gels and measured for radioactivity using a Beckman Scintillation Counter LS 6500.

**RESULTS**

**RPA ssDNA Binding Activity Is Regulated by Redox**—In an effort to understand the regulatory function of RPA, we examined whether RPA ssDNA binding activity is affected by redox. 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. RPA-DNA complex was analyzed by electrophoretic mobility shift assay on polyacrylamide gel under equilibrium conditions (Fig. 1a and data not shown). A very low RPA-DNA complex was formed in the absence of DTT, which was stimulated up to 10-fold by the addition of DTT (Fig. 1a). To examine redox regulation further, RPA was treated with the oxidizing agent diamide in the presence of 0.4 mM DTT. The addition of increasing amounts of diamide gradually decreased the formation of RPA-DNA complex (Fig. 1b). The inhibitory effect of diamide (3.2 mM) on RPA-DNA interaction was reversed by DTT only when added more than the stoichiometric amounts (Fig. 1c; lanes 7–8). These results strongly suggest that RPA ssDNA binding activity is regulated by redox potential.

**Effect of Reducing Agents on RPA-ssDNA Interaction**—We then examined how redox potential affects RPA DNA binding affinity. In the absence of DTT, RPA ssDNA binding activity was sensitive to NaCl, whereas under reducing conditions (1 mM DTT), RPA formed a stable complex with ssDNA even in the presence of 1.0 mM NaCl (Fig. 2a). This result suggests that redox affects RPA ssDNA binding affinity such that RPA-ssDNA interaction was significantly enhanced under reducing conditions. Thioredoxin and redox factor-1 (ref-1, also known as apurinic/apyrimidinic endonuclease) have previously been shown to mediate redox regulation of nuclear proteins such as transcription factor, activator protein-1, and glucocorticoid receptor (38, 39). As part of an effort to identify a cellular factor(s) involved in redox regulation of RPA, we also examined whether RPA DNA binding activity can be modulated by thioredoxin, a ubiquitous redox enzyme involved in the formation of reversible disulfide bonds (40). Incubation of RPA with thioredoxin stimulated RPA-ssDNA complex under conditions where RPA itself or with the buffer (without thioredoxin) showed no DNA interaction.
Redox Regulation of Human Replication Protein A

FIG. 2. Effect of reducing agents on RPA ssDNA binding activity. a, titration of NaCl on RPA ssDNA binding activity in the presence and absence of DTT. Wild-type RPA (20 ng) was treated with 0 mM (lanes 2–8) or 1 mM DTT (lanes 9–15) for 15 min at room temperature before incubation for 15 min with 100 fmol of 5'32P-labeled oligo(dT)50 in the presence of 0 mM (lanes 2 and 9), 25 mM (lanes 3 and 10), 50 mM (lanes 4 and 11), 100 mM (lanes 5 and 12), 200 mM (lanes 6 and 13), 500 mM (lanes 7 and 14), or 1000 mM (lanes 8 and 15) of NaCl. After the reactions, the RPA-DNA complex was analyzed by the procedure described in Fig. 1a. b, effect of redox protein, thioredoxin, on RPA DNA binding activity. Wild-type RPA (20 ng) was incubated with an increasing amount (2 and 4 μl) of DTT-containing buffer (20 mM Hepes-KOH, pH 7.8, 50 μM DTT, 60 mM KCl, 0.1 mM EDTA, and 5% glycerol) (lanes 3 and 4, respectively) or the same buffer containing 75 μg (lanes 5) or 150 ng (lanes 6 and 7) of thioredoxin. Lane 8 contained 2 mM DTT instead of thioredoxin. After 15 min at room temperature, 100 fmol of 5'32P-labeled oligo(dT)50 was added to the reactions mixtures and subsequently analyzed for RPA-DNA complex as described in Fig. 1a.

binding activity (Fig. 2b), suggesting that RPA DNA binding activity may be regulated by a redox protein in vivo.

RPA DNA Binding Activity Is Inhibited by a Sulfhydryl Group Modifying Agent, N-ethylmaleimide. Where indicated, 10 ng (lanes 2, 5, and 8), 20 ng (lanes 3, 6, and 9), or 40 ng (lanes 4, 7, and 10) of RPA was used. Various amounts of RPA were treated with either 2 mM DTT (lanes 2–4), 5 mM NEM (lanes 5–7), or 50 mM DTT followed by 5 mM NEM (lanes 8–10) before incubation with 100 fmol of 5'32P-labeled oligo(dT)50. The RPA-DNA complex was analyzed by gel mobility shift assay as described in Fig. 1a.

NEM on RPA DNA binding activity is due to the alkylation of sulfhydryl group (Fig. 3, lanes 8–10). The inhibitory effect of NEM on RPA ssDNA binding activity strongly indicates that cysteine residues are involved in the redox regulation of RPA DNA binding activity.

Involvement of Zinc Finger Cysteine(s) in Redox Regulation of RPA—RPA p70 contains an evolutionarily conserved 4-cysteine-type zinc finger domain at amino acids 478–503 (28). Even though previous studies showed that the zinc finger domain is not essential for DNA binding activity of RPA (3–5), it is possible that RPA DNA binding domain is subjected to regulation by the zinc finger. We therefore examined whether the cysteine residues of the zinc finger domain are involved in redox regulation of its ssDNA binding activity. In contrast to wild-type RPA, zinc finger mutant-4 (ZFM-4; Cys to Ala mutation at amino acids 481, 486, 500, and 503) formed a stable complex with ssDNA even under nonreducing conditions, and the addition of DTT had no effect on its DNA binding activity (Fig. 4c). Two other zinc finger mutants, ZFM-1 (Cys to Ala at amino acid 486) and ZFM-2 (Cys to Ala mutation at amino acids 481 and 486), also formed a very stable complex with ssDNA in the absence of DTT, which was not affected by DTT (Fig. 4b). To further examine redox regulation of zinc finger domain, wild-type RPA and ZFM-4 were treated with the oxidizing agent, H2O2, in the presence of 0.4 mM DTT (Fig. 4e). The addition of increasing amounts of H2O2 significantly reduced the RPA-DNA complex, whereas zinc finger mutant (ZFM-4) (1) was much less affected by H2O2 treatment (Fig. 4e). These results strongly suggest that the zinc finger is involved in the redox regulation of RPA ssDNA binding activity and that the cysteine residues of p70, in particular cysteine 486, are essential for this regulation.

Zn(II) Is Necessary for Redox Regulation of ssDNA Binding Activity—If RPA is a Zn(II) metalloprotein and Zn(II) is bound to the 4-cysteine complex, the presence of Zn(II) would be able to protect these cysteines from oxidation. To test this, we examined whether a strong divalent cation chelator, o-phenanthroline, affects RPA ssDNA binding activity in response to
FIG. 4. Cysteine residue(s) of zinc finger is involved in redox regulation of RPA ssDNA binding activity. a, comparison between wild-type (W.T.) RPA and zinc finger mutant (ZFM-4) for ssDNA binding activity in the presence of varying concentrations of DTT. Twenty ng of either wild-type RPA (lanes 2–6) or ZFM-4 (lanes 7–11) were pretreated with 0 mM (lanes 2 and 7), 0.02 mM (lanes 3 and 8), 0.2 mM (lanes 4 and 9), 2 mM (lanes 5 and 10), and 20 mM (lanes 6 and 11) DTT and further incubated for 15 min at room temperature after the addition of 100 fmol of 5'-32P-labeled oligo(dT)50. The RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 79:1). b, Twenty ng of either wt-RPA (lanes 2–3) or ZFM4 (lanes 4–5) were pretreated with 1 mM DTT (lanes 2–3) or 1.0 mM o-phenanthroline (lanes 4 and 5) and further incubated for 15 min. After the addition of 100 fmol of 5'-32P-labeled oligo(dT)50, the RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis. c, Twenty ng of either wt-RPA (lanes 2–4) or ZFM4 (lanes 5–7) were pretreated with 0 mM (lanes 2–4 and 5–7) or 1.0 mM Zn(II) (lanes 3–4 and 5–7) and further incubated for 15 min at room temperature after the addition of 100 fmol of 5'-32P-labeled oligo(dT)50. The RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis.

FIG. 5. Effect of Zn(II) chelating agent, o-phenanthroline, on RPA ssDNA binding activity. a, increasing amounts of wt-RPA (5, 10, 15, and 20 ng, respectively) (lanes 2–13) were pretreated with 0 mM DTT (lanes 2–5), 1.0 mM DTT (lanes 6–9), or 1.0 mM of DTT and o-phenanthroline (lanes 10–13) and further incubated for 15 min at room temperature after the addition of 100 fmol of 5'-32P-labeled oligo(dT)50. No RPA was included in lane 1. The RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 79:1). b, Twenty ng of either wt-RPA (lanes 2–3) or ZFM4 (lanes 4–5) were pretreated with 1 mM DTT (lanes 2–3) or 1.0 mM o-phenanthroline (lanes 4 and 5) and further incubated for 15 min. After the addition of 100 fmol of 5'-32P-labeled oligo(dT)50, the RPA-DNA complex was analyzed by 5% polyacrylamide gel electrophoresis. No RPA was included in lane 1.

**DISCUSSION**

Zinc fingers are not only autonomously folding structural elements but also are the DNA binding component for many
sequence-specific DNA binding proteins and nuclear hormone receptors (46). A number of zinc finger proteins have been identified in which their DNA binding activity is regulated by redox, although the role of the zinc finger in this regulation is not clear (47–49). In this study, we found that RPA ssDNA binding activity is regulated by redox through the cysteines in a putative zinc finger domain.

The 4-cysteine type zinc finger is evolutionarily conserved among eukaryotic RPA and contains highly conserved hydrophobic and charged amino acids that may be important for the formation of zinc finger structure (Fig. 6a). The 4-cysteine zinc finger contains Zn(II), which tetrahedrally coordinates four cysteine residues (50). Under reducing conditions, the zinc finger structure is favorably formed, and Zn(II), buried in the interior, stabilizes the module by binding 4 cysteines (Fig. 6b). This zinc finger structure protects cysteine 486 (and other cysteine residues) from being engaged in the formation of disulfide bond(s). Under nonreducing (or oxidized) conditions, however, oxidation of Zn(II)-thiolate bond induces the releases of Zn(II) from the zinc finger (51), which promotes the formation of disulfide bonds between the cysteine 486 and other cysteine (Fig. 6b). It is reasonable to assume that one disulfide bond is formed between cysteine 486 and the other cysteine, because the Cys to Ala mutation at amino acid 486 was sufficient enough to make it redox-insensitive (Fig. 6b). In fact, our study on the reactivity of cysteine residues to 5,5′-dithiobis(2-nitrobenzoic acid) indicated that two zinc finger-derived cysteines are lost on oxidation (data not shown), supporting a model that a single disulfide bond is formed in the zinc finger domain. Disulfide bonds may be formed between cysteine 486 and the other cysteine in the zinc finger region, which induces a structural change that interferes with the DNA binding domain of p70 (Fig. 6b). Alternatively, a disulfide bond may be formed between cysteine 486 and the other cysteine outside of the zinc finger, which leads to the alteration of protein conformation that affects RPA DNA binding activity. To understand the role of zinc finger further, a direct measurement of the actual release of Zn(II) from RPA upon oxidation by atomic absorption or by spectrophotometric measurement using 4-(2-pyridylazo)resorcinol needs to be done once we have enough RPA.

In T4 bacteriophage, removal of the intrinsic Zn(II) ion in its single-stranded DNA-binding protein (T4 gene 32) resulted in facile oxidation of the cysteine, which significantly decreased ssDNA binding activity (52). In T4 gene 32, models for the oxidized and metal-free protein are essentially inactive while reduced, and fully metal-bound forms are active in ssDNA binding (52, 53). However, the substitutional mutation of metal binding cysteine to serine in T4 gene 32 was essentially inactive in ssDNA binding (52), whereas a zinc finger mutation of human RPA (single-stranded DNA-binding protein) made it redox-insensitive without affecting its ssDNA binding activity (Fig. 4). These results suggest that Zn(II) binding cysteine(s) of T4 gene 32 is an essential element for its ssDNA binding, but that of human RPA functions as a regulatory element.

Redox regulation of RPA likely occurs in vivo because RPA is involved in replication arrest in response to environmental stress (33, 34). Even though the formation of disulfide bond is favorable under oxidized conditions, it is still possible that cysteine 486 exists another oxidized form in vivo. For example, involvement of the glutathione/glutathione disulfide redox pair, an important cellular defense against oxidative stress (55), is considered a possibility. Another example of oxidative signaling is S-nitrosylation of cysteine residues in p21WAF1/cip1 oncogene by nitric oxide (56), even though it is unclear whether this is a reversible intercellular-signaling scheme.

Zinc finger proteins are one of the largest classes of DNA-binding proteins and are the major targets for redox regulation since they all contain cysteine residues as part of the metal binding finger structure, which are essential for DNA binding activity (46). Cysteine residues have been identified in several non-zinc finger transcription factors as involved in redox regulation of their DNA binding activity (42, 57). However, it is not clear whether the zinc finger itself is directly involved in redox regulation simply because the zinc finger is an essential DNA binding component and any mutation at a cysteine residue (of zinc finger) will irreversibly inactivate DNA binding activity. Unlike these zinc finger proteins, the RPA zinc finger is not a DNA binding component and has a little or no effect on its DNA binding activity (1, 3–5), which makes it an excellent model to study the role of redox in regulation of zinc finger protein. We conclude from this study that the RPA zinc finger has a role in regulating its DNA binding activity through redox change.

\[ a \]

- Human
- Xenopus l.
- Drosophila
- S. pombe
- S. cerevisiae
- C. elegans
- C. fasciculata

\[ b \]

- Redox-induced change in zinc finger structure and regulation of RPA DNA binding activity. a, zinc finger domain (X,CX2CX3CX6X,CX6 of eukaryotic RPA. Conserved amino acids are indicated in bold-type (cysteines), boxed (hydrophobic residues), and underlines (charged residues). b, a stable zinc finger structure is formed under reducing condition. Under oxidizing condition, Zn(II) is released from the zinc finger, which induces the formation of disulfide bond(s) between cysteine residues, including 486. The open bar indicates the RPA DNA binding domain. Xenopus l., Xenopus laevis; S. pombe, Schizosaccharomyces pombe; S. cerevisiae, Saccharomyces cerevisiae; C. elegans, Caenorhabditis elegans; C. fasciculata, Crithidia fasciculata.
Redox Regulation of Human Replication Protein A

which may be involved in regulation of DNA metabolism in response to various environmental stress.

Acknowledgements—We thank Drs. M. Kelley and D. Giedroc for the suggestions during the course of experiments, Dr. D. Ohanesian for critical reading of the manuscript, and E-J. Oh for the art work.

REFERENCES

1. Dong, J., Park, J-S. & Lee, S-H. (1999) Biochem. J. 337, 311–317
2. Lin, Y.-L., Shoji, M. K. R., Chen, C., Kolodner, R., Wood, R. D. & Dutta, A. (1996) Mol. Cell. Biol. 16, 4979–4988
3. Sugiyama, T., New, J. H. & Kowalczykowski, S. C. (1998) Nature 391, 497–510
4. Hays, S. L., Firmenich, A. A., Massey, P., Banerjee, R. & Berg, P. (1998) Mol. Cell. Biol. 18, 4400–4406
5. Gomes, X. V. & Wold, M. S. (1995) J. Biol. Chem. 270, 4534–4543