Identification of the Structural Change of Human Replication Protein A (hRPA) in the ssDNA Binding and Redox Potential

Suhkmann Kim,[†] Andre Kim, Chun-Ri Li, Shin-Won Kang, and Jang-Su Park[*]

Department of Chemistry and Centre for Innovative Bio-physio Sensor Technology, Pusan National University, Busan 609-735, Korea. [*]E-mail: jaspark@pusan.ac.kr
[†]National Research Laboratory for Computational Proteomics and Biophysics, Department of Physics, Pusan National University, Busan 609-735, Korea
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Eukaryotic replication protein A (RPA) is comprised of a three subunits (70, 32, 14 kDa; p70, p32, p14 respectively). RPA has been identified as a human protein necessary for SV40 DNA replication *in vitro*[1-3] and acting as a single-strand DNA (ssDNA) binding protein with multiple functions in DNA replication, repair, and genetic recombination.[1-5]

Recent studies suggest that RPA functions are regulated in response DNA damage[6] and that its ssDNA binding activity may be involved in this regulatory event.[7] In our previous study, we found that RPA's ssDNA binding activity is regulated by redox through cysteins in the putative zinc finger domain of the p70 subunit.[8,9] A number of DNA-binding proteins have been identified in which their DNA binding activity is regulated by redox, although the regulatory role of the zinc finger domain is not clear.

RPA70 has an evolutionarily conserved 4 cystein in the zinc-finger domain at 473 through 503 amino acids. Even though previous studies showed that the zinc-finger domain does not participate in DNA binding directly, it is still possible that the zinc-finger domain regulates DNA binding activity. To verify this possibility, we built mutant RPA (ZFD4) by substitution four cysteins for alanine and checked the DNA binding activity. The ZFD4 formed a stable complex with the ssDNA, even under oxidized conditions, and the amounts of DTT had no effect on its binding property (Figure 1a). The ZFD4 also showed strong binding activity in the non-reducing condition without DTT. The addition of H_2O_2 did not have much of effect on RPA-DNA complex formation (Figure 1a), whereas the wild-type RPA had H_2O_2 dependent activity.[7-9] These results suggest that the zinc-finger domain could regulate the ssDNA binding activity of RPA and that the cystein residues of the p70 subunit are essential in this process. We examined the effect of phosphorylation on ZFD4's in regulation of ssDNA binding activity of RPA in the various redox conditions. The results showed phosphorylation of ZFD4 does not affect its DNA binding activity and suggests that phosphorylation could occur at other sites of the RPA complex (Figure 1a).

In an effort to understand RPA's regulatory function, we examined the structural changes of hRPA by the ssDNA binding using protease sensitive digestions. We examined

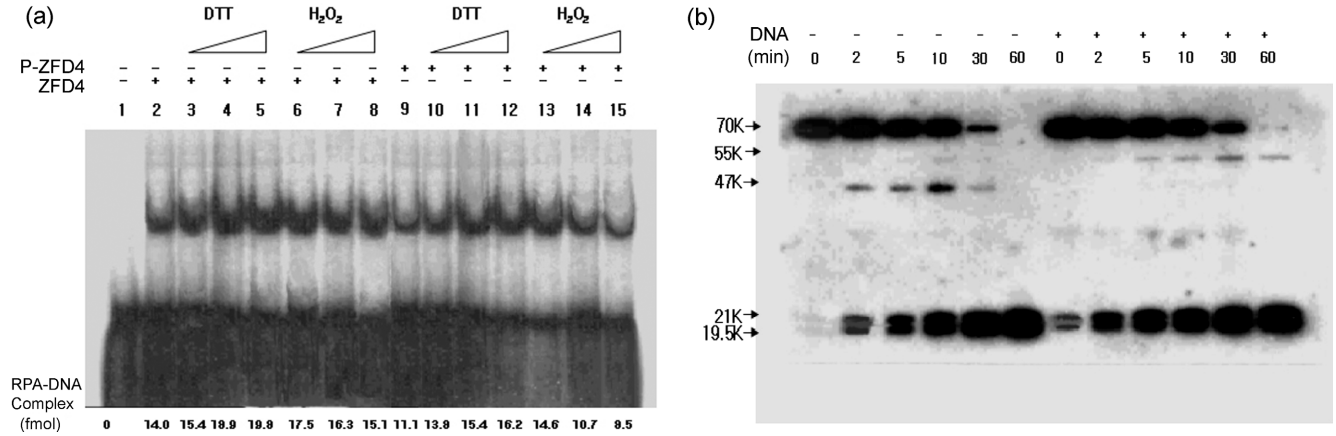

Figure 1. (a) The ssDNA binding activity of ZFD4 and phosphorylated ZFD4. The indicated amounts of DTT or H_2O_2 were added to the mixture and the mixtures were incubated at room temperature. RPA-DNA complexes were analyzed by 5% polyacrylamide gel electrophoresis (acrylamide: bisacrylamide = 79 : 1). The interested bands were excised from the gels and their radio activities were detected using Beckaman Scintillation Counter LS6500. (b) Effect of zinc-finger domain on conformational change of wild-type p70. Reaction mixtures contained 0.1 M Tris-HCl (pH 8.5) and 120 ng/μL of wild-type RPA. 40 ng/μL of oligo(dT)$_{50}$ was added. After incubation at room temperature for 15 min, 50 ng of trypsin was added. Then the mixtures were incubated at 37 °C and aliquots were removed from the reactions at the indicated time point. The samples were mixed with a gel loading buffer, boiled for 5 min and loaded onto 14% SDS-PAGE. The gel was then subjected to a western blot analysis using an anti-p70 polyclonal antibody.

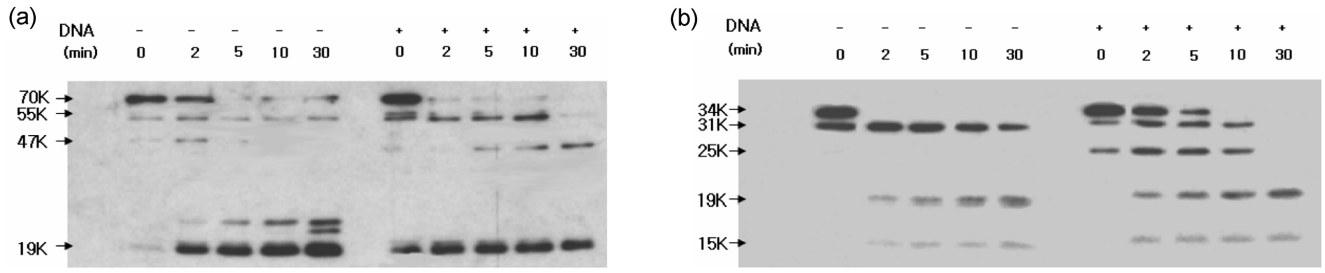

Figure 2. (a) and (b) are effects of zinc-finger domain on conformational change of ZFD4 p70 and wild-type p32. The experimental methods are the same with Figure 1(b).

the structure of hRPA by analyzing its immunoblot patterns. Figure 1b shows the cleavage pattern of hRPA70 at various time periods of digestion by trypsin. Trypsin rapidly degraded hRPA into distinct proteolytic 47, 21 and 19.5 kDa fragments (Figure 1b).

Next, we examined whether the interactions with ssDNA binding changed the sensitivity of hRPA to the protease digestion. Figure 1b shows the cleavage of hRPA70 by trypsin in the absence or presence of oligo(dT)$_{50}$. When ssDNA was absent, the internal ~55 kDa fragment degraded to the point of disappears in less than two minutes and a ~47 kDa band appeared. When ssDNA was present, the ~55 kDa fragment was gradually digested. From the digestion pattern, we concluded that the binding of RPA to ssDNA decreases the rate of proteolytic cleavage of hRPA70 and resulted in the appearance of at least one additional trypsin cleavage site. These changes could either be caused by the bound ssDNA sterically blocking access of protease or by a DNA-induced conformational change of the hRPA70 itself. We also examined the digestion of ZFD4 at various time periods by trypsin. Figure 2a shows its cleavage pattern in the absence or presence of oligo(dT)$_{50}$. When ssDNA was present, the ZFD4 became much more resistant to digestion by trypsin and the ~55 kDa fragment resisted further digestion. In contrast, when ssDNA was absent, the internal ~55 kDa fragment was gradually digested and became a ~25 and ~21 kDa fragment.

Then, we examined that the proteolysis pattern of subunit p32 and the role of subunit p32 in ssDNA binding. After treatment of trypsin, the initial digestion of p32 subunit generated a ~30 kDa fragment (Figure 2b).

This truncated polypeptide was relatively resistant to further cleavage by trypsin. From the investigation of the sequence in the presence of ssDNA, RPA32 became more sensitive to trypsin digestion and the ~30 kDa fragment was rapidly degraded to 25, 19 and 15 kDa fragments. We concluded that the N-terminal residues of RPA p32 are sensitive to proteolytic cleavage and the N-terminus of p32 is either on the surface of the complex or on the extended conformation. That the binding RPA to ssDNA caused more proteolytic cleavage on the p32 suggests that the conformational change could be due to the ssDNA binding. We also tested ZFD4 and wild type p32 complex to confirm the role of the ssDNA binding effect on the digestion of the p32. The results showed that the ssDNA binding did not affect proteolytic digestion of the mutant complex (Figure 2a) and suggested that the ssDNA binding of the p70 subunit induced a structural change in the hRPA complex or the ssDNA binding could occur at the p32 subunit when the p70 subunit was occupied by ssDNA.

We confirmed the structural changes to the wild type hRPA and ZFD4 in the different redox conditions (Figure 2b) with Circular Dichroism (CD) spectroscopy. The wild type hRPA showed different spectra patterns in the oxidizing and reducing states. This result suggests that there is a secondary structural change in the RPA by the redox condition and could explain our previous report which indicated that the wild type hRPA is regulated by the redox potentials in its binding with ssDNA.[9] In contrast with the wild type hRPA, ZFD4 showed similar spectra in the oxidizing and reducing conditions. This result suggests that the secondary structures of ZFD4 were not changed by the redox states and the four missing cysteins have an important role in its redox susceptibility.

The p70 and p32 subunits were affected by its structure in DNA binding. These results are surprising because the p32 subunit is not known to participate in DNA binding activity and the further investigation is required to clarify the structural changes in atomistic level.

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