DOI 10.5012/bkcs.2009.30.12.2867

Zn^{2+}-Dependent Single-Stranded DNA Binding and DNA Replication Activities in Replication Protein A

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Received September 1, 2009, Accepted October 6, 2009

Key Words: Replication Protein A, Redox, Single-stranded DNA-binding, DNA replication, Zinc-finger motif

Replication Protein A (RPA) is a heterotrimeric (70-, 32-, and 14-kDa subunits) protein containing six oligonucleotide binding folds (OB-folds),[1-3] which are referred to as DNA-binding domains (DBD-N, -A, -B, -C, -D, and -E) (Fig. 1A).[2] Recently, we performed the PONDR (Predictors of Natural Disordered Regions) program to predict naturally disordered regions of human RPA 70 subunit. The x axis in Fig. 1B shows the residue number, whereas the y axis is the PONDR score normalized to be in the range of 0 ~ 1. Any region that exceeds a score of 0.5 is considered to be disordered. A four cysteine-type (C^{481}-X_4-C^{486}-X_{13}-C^{500}-X_2-C^{503}) (arrow on the right) zinc-finger domain (ZFD) shows a most stable and structured segment in RPA70 subunit (its PONDR score is close to 0). Previous studies reveal that DBD-C, at the C terminus of RPA70, is required for the formation of the heterotrimeric core complex (with the RPA-32 and RPA-14 subunits) (Fig. 1A),[6,7] and the ZFD regulates the ssDNA-binding activity of RPA through a redox change.[8,9] Recently, analysis by liquid chromatography/tandem mass spectrometry revealed that two pairs of intramolecular disulfide bonds (Cys^{481}-Cys^{486} and Cys^{500}-Cys^{503}) are formed under oxidative conditions.[10] Interestingly, our PONDR prediction showed that the replacement of the cysteine amino acids (ZFM-RPA; Cys to Ser at 481, 486, 500, and 503) results in the ZFD being in a flexible and unstable state (right panel in Fig. 1B). Thus, the breakdown of the zinc-finger motif could influence the stabilization of DBD-C, subsequently changing the activity of the whole RPA complex.

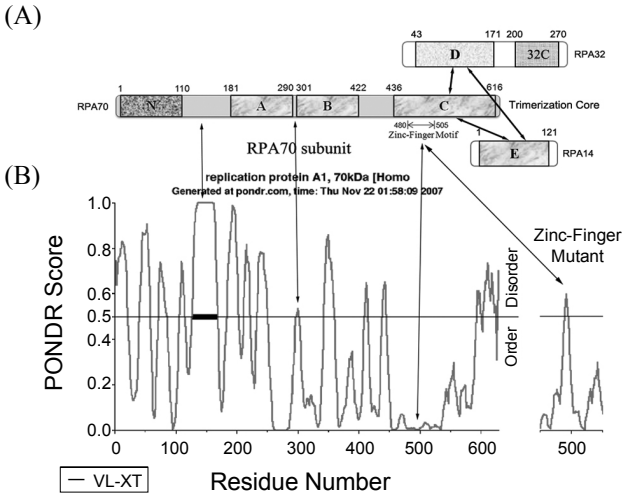

Figure 1. (A) A schematic of the heterotrimer complex of human RPA. Six OB-fold domains were identified, which are referred to as the DNA-binding domains (DBD -N, -A, -B, -C, -D, and -E). (B) Disorder prediction of the hRPA70 subunit by PONDR. The x axis shows the residue number, whereas the y axis is the PONDR score normalized to be in the range of 0 ~ 1. Any region that exceeds a score of 0.5 is considered to be disordered. The PONDR score of the segment (residues 110 - 181, arrow on the left), between DBD-N and -A, is above 0.5. The PONDR score of the ZFD (residues 480 - 505), which contains a four cysteine-type (C^{481}-X_4-C^{486}-X_{13}-C^{500}-X_2-C^{503}) motif, is close to 0 (arrow on the right). The ZFM showed a disordered and unstructured region (right panel in Fig. 1B).

To obtain the native RPA proteins, we expressed a constructed pET11a vector encoding human RPA cDNA in *E.coli* BL21 (DE3) strains using TB medium.[11] After adding IPTG to TB medium, the RPA32 and RPA14 (but not RPA 70) subunits could be well expressed (Fig. 2, asterisk in lane 2). Considering that TB medium may not contain the enough Zn(II)-ion,[12] 10 μM of $ZnCl_2$ was added to TB medium throughout all the RPA induction (lanes 3-4). The yield of the induced RPA70 subunit was greatly increased, when $ZnCl_2$ was added (asterisk in lane 4). We think whether Zinc-lacking results in the failure of zinc-finger motif formation in RPA70 subunit (but forming an oxidative

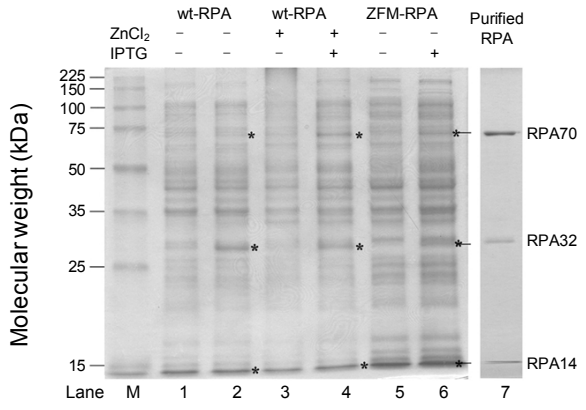

Figure 2. The effect of Zn(II) on the bacterial expression of hRPA in TB medium. The Zn(II)-ion was in the absence of cell-culture mediums (lanes 1, 2, 5, and 6) of RPA.

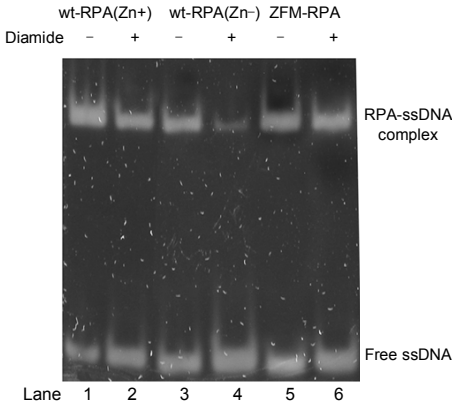

Figure 3. Single-stranded DNA-binding assays of wt-RPA and its ZFM-RPA mutants. The RPAs (40 ng) were firstly treated (or not) by the indicated amount of diamide (20 minutes, 20 °C) and then incubated with 1 pmol of oligo $(dT)_{50}$ at room temperature for 15 min. The RPA-ssDNA complexes were analyzed using 5% native-polyacrylamide gels.

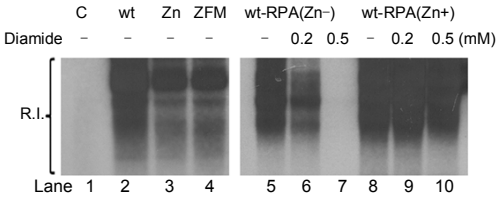

Figure 4. DNA replication assays of wt-RPA and its ZFM-RPA mutants. The assay was performed as described previously with minor modifications.[14]

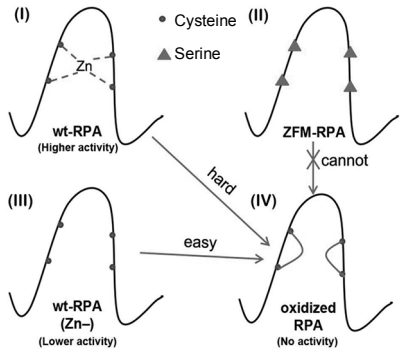

Figure 5. A schematic mechanism of the requirement for Zn(II) in the zinc-finger domain (ZFD) for full function of RPA. The wt-RPA containing Zn(II)-ion exhibits the highest ssDNA-binding and DNA replication activities (form I). When the ZFD is disrupted (form II and form III), the RPA complexes show somewhat weaker activities than those in the form-I condition. Meanwhile, the wt-RPA from which the Zn(II)-ion is released (form II) is sensitive to oxidative stress and easily forms two pairs of disulfide bridges (form IV).

state[10] of two pairs of intramolecular disulfide bonds [Cys^{481}-Cys^{486}] and [Cys^{500}-Cys^{503}]), which is a toxic form of RPA70 subunit), Meanwhile, we expressed a ZFM-RPA in the Zinc-lacking TB medium and found that the mutated RPA70 subunit was well-induced by IPTG (lanes 5-6). We then investigated whether Zn(II) is required for the ssDNA-binding activity of RPA. The same amounts of three RPA samples (wild-type RPA (wt-RPA), wt-RPA(Zn-), and ZFM-RPA) were examined by the electrophoretic mobility shift assay.[9,14] In Fig. 3, the wt-RPA complex exhibited a very high ssDNA-binding activity under reductive conditions (lane 1). This activity was decreased, but not entirely inhibited, in the presence of 0.5 mM diamide (DM; an oxidizer) (lane 2). Meanwhile, wt-RPA(Zn-) showed weaker ssDNA-binding activity than wt-RPA, and this activity was inhibited by DM (lanes 3-4). These results suggest that the release of Zn(II) from the ZFD decreases the ssDNA-binding activity, but the presence of Zn(II) is able to protect these cysteines from forming oxidized disulfide bonds. Furthermore, ZFM-RPA showed similar activities (but weaker than those of wt-RPA) under the above two conditions (lanes 5-6). This result reveals that disruption of ZFD influences the structure of the whole RPA complex, and ZFM-RPA could not be regulated by redox changes. To investigate whether the Zn(II) ion influences the effect of RPA on DNA replication, the above three RPA samples were also examined by the *in vitro* SV40 DNA replication assay. In keeping with these previous studies, all three RPA samples efficiently supported SV40 DNA replication (Fig. 4, lanes 2-4), except that the replication activities with wt-RPA (Zn-) and ZFM-RPA were somewhat lower than those with wt-RPA. Furthermore, wt-RPA(Zn-) lost its DNA replication activity in the presence of DM, however wt-RPA containing the Zn(II)-ion was resistant to this oxidative stress (lanes 5-10).

In conclusion, the findings of this study support the conclusion (Fig. 5) that the RPA complex lacking the Zn(II)-ion shows lower ssDNA-binding and DNA replication activities than that containing this divalent, metallic ion. Meanwhile, the release of the Zn(II)-ion results in the activities of the RPA-complex becoming sensitive to oxidative damage.

Acknowledgments. This work was supported by Pusan National University Research Grant (Two years, 2009-2010), and by the Basic Research Program (KOSEF-2009-0064961) of Korea Science and Engineering Foundation.

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