Investigation of the core binding regions of human Werner syndrome and Fanconi anemia group J helicases on replication protein A

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Werner syndrome protein (WRN) and Fanconi anemia group J protein (FANCJ) are human DNA helicases that contribute to genome maintenance. They interact with replication protein A (RPA), and these interactions dramatically enhance the unwinding activities of both helicases. Even though the interplay between these helicases and RPA is particularly important in the chemoresistance pathway of cancer cells, the precise binding regions, interfaces, and properties have not yet been characterized. Here we present systematic NMR analyses and fluorescence polarization anisotropy assays of both helicase-RPA interactions for defining core binding regions and binding affinities. Our results showed that two acidic repeats of human WRN bind to RPA70N and RPA70A. For FANCJ, the acidic-rich sequence in the C-terminal domain is the binding region for RPA70N. Our results suggest that each helicase interaction has unique features, although they both fit an acidic peptide into a basic cleft for RPA binding. Our findings shed light on the protein interactions involved in overcoming the DNA-damaging agents employed in the treatment of cancer and thus potentially provide insight into enhancing the efficacy of cancer therapy.
mouse WRN contains only one acidic-rich sequence (27 a.a), highly conserved with human WRN, and because it was revealed that 15 a.a. of the acidic-rich region was sufficient to support RPA70N binding,16–18 we wondered what purpose two acidic repeats might serve in the human WRN-RPA interaction. In order to define the core binding sequence and investigate the binding properties, we tested the binding of various WRN constructs to RPA70N and RPA70A with nuclear magnetic resonance (NMR) spectroscopy and fluorescence polarization anisotropy (FPA) assays.

It has been reported that FANCJ co-localizes with RPA in nuclear foci that contain BRCA1 after DNA damage.11 It was also revealed that interaction with RPA restores the unwinding activity of FANCJ, which can be blocked by TTAGGG repeat binding factors in the forked duplex telomeric substrate.20 These results show that the FANCJ-RPA interaction plays an essential role in DNA metabolism, including replication and repair. However, the details of the binding properties, such as the binding region of each partner, have not been studied. Interestingly, FANCJ is the only member of the human 2 Fe-S helicase superfamily that physically interacts with RPA70,11 and unlike other family members, FANCJ has an unstructured C-terminal region which contains acidic-rich sequences. Two acidic sequences in the C-terminus of FANCJ (residues 1120–1133 and 1201–1213) have similar sequence compositions to known RPA70N interactors such as p53,21 ATRIP,17 BLM,18 SV40,22 and ETAA,123. Based on this, we hypothesized that acidic regions of the C-terminus of FANCJ could interact with RPA70N, and we tested this hypothesis with NMR spectroscopy and FPA assays.

In this study, we performed chemical shift perturbation (CSP) analyses of RPA70N and RPA70A using titrations of various constructs from WRN and FANCJ helicases. We mapped the binding interfaces and measured dissociation constants of each binding pair using FPA assays. We found that the acidic WRN peptide binds not only to RPA70N but also, weakly, to RPA70A. Our FPA analysis showed that the two tandem acidic repeats bind to a dual RPA70N-A construct tighter than the single acidic peptide binds to RPA70N alone. We also found that FANCJ1120–1133 specifically interacts with RPA70N, and that two aromatic residues of the sequence are crucial for the binding. Our analysis provides detailed information on the WRN-RPA and FANCJ-RPA interactions that may inform inhibitory strategies for each helicase.

Results
Chemical shift perturbation analysis of RPA70N upon binding to WRN422–484. Previous studies have shown that two acidic peptide repeats of WRN located in the N-terminus (WRN424–475, 52 a.a.) mainly interact with RPA70.10,12. We first performed a series of 1H-15N HSQC experiments for the CSP analysis of 15N-labeled RPA70N with WRN422–484 (64 a.a.) to confirm WRN-RPA70N binding. Figure 2A shows the overlaid 1H-15N HSQC spectra of 15N-labeled RPA70N in the absence or presence of increasing molar ratios of WRN422–484. Several peaks gradually shifted upon the addition of WRN422–484. In the graph of the average CSPs (Δδavg) of RPA70N upon binding to WRN422–484, T34, T35, L45, S55, F56, V94, and E120 were perturbed by more than two standard deviations above the average (Fig. 2B). Figure 2C shows those residues in red and residues with Δδavg greater than one standard deviation above the average in green on the crystal structure of RPA70N (PDB ID: 2B29).24 The region is largely overlapped with the basic cleft of RPA70N, which is responsible for the binding of several DNA damage response proteins. Perturbation of the isolated E120 in the flexible C-terminal end is likely due to allosteric effects of peptide binding rather than direct interaction.
Binding affinity of WRN peptides for RPA70N and RPA70A. Our CSP analysis showed that WRN422–484, a region containing two acidic peptide repeats, interacts with RPA70N. In order to define the core region of WRN for RPA binding and examine whether RPA70A also binds to WRN, we performed FPA assays of three FITC-tagged peptides—WRN435–450, WRN426–436, and WRN441–450—upon addition of increasing concentrations of RPA70N or RPA70A (Fig. 3A). We obtained the Kd value for each peptide upon titration with RPA70N (Fig. 3B) or RPA70A (Fig. 3C). Table 1 shows the Kd values of all the samples we tested. All three peptides bound to RPA70N with Kd values in the micromolar range, while they showed weaker interactions with RPA70A than RPA70N. WRN441–450, which solely contains acidic residues, showed the lowest Kd (29.3 ± 1.2 μM) for RPA70N. Interestingly, the lowest Kd for RPA70A (65.2 ± 8.1 μM) was observed with WRN435–450. This is 1.5-fold weaker than its binding to RPA70N. Our results showed that a single acidic repeat (WRN435–450) could bind to both RPA70N and RPA70A with different affinities. This raises the possibility that the first acidic repeat binds to RPA70N and the other repeat binds to RPA70A or vice versa. FPA assays of FITC-tagged WRN435–476 upon addition of increasing concentrations of RPA70N-(GGGGS)2-RPA70A (RPA70N-A) protein was performed to confirm this possibility (Fig. 3D). The Kd value of this case was estimated as 14.9 ± 5.8 μM (Table 1). This result showed that the two acidic repeats (WRN435–476) could bind to RPA70N-A 2-fold stronger than WRN441–450 - RPA70N binding.

Mapping of the WRN435–450 peptide binding surface on RPA70N and RPA70A. Our FPA assays showed that WRN435–450 could interact with both RPA70N and RPA70A. In order to map WRN binding surfaces on RPA70N and RPA70A, we performed 1H-15N HSQC titrations with the WRN435–450 peptide. The final pH of the WRN435–450:RPA samples at a 2:1 molar ratio was 7.14 because of residual TFA. However, we confirmed that the chemical shifts of the backbone amide protons of RPA70N are almost the same as those at pH 7.4 (Supplementary Fig. S1).

Supplementary Fig. S2 shows the overlaid 1H-15N HSQC spectra of RPA70N and 1H-15N cross-peaks of S55, M57, R92, and E120 of RPA70N upon titration with WRN435–450. Our results showed S55, M57, N85, R92, and E120 were perturbed by more than two standard deviations above the average (see Supplementary Fig. S2). T34, T35, and L45, which were significantly perturbed by WRN422–484, did not have a significant shift change. Even though the absolute magnitudes of Δδavg were reduced compared to WRN422–484 titration, WRN435–450 still specifically interacted with the basic cleft region of RPA70N.

In the case of RPA70A, Δδavg values were small, and the specifically perturbed residues (W212, N214, G219, K220, and E240) were largely overlapped with the ssDNA binding region (see Supplementary Fig. S2). Also, the binding surfaces were similar to the Rad51-RPA70A interaction.

Chemical shift perturbation analysis of RPA70N upon binding to FANCJ1120–1211. In order to examine our hypothesis that the C-terminal region of FANCJ could specifically engage RPA, we monitored CSPs of the backbone amide peaks of RPA70N in 1H-15N HSQC spectra upon addition of FANCJ1120–1211. As shown in Fig. 4A,B, FANCJ1120–1211 mainly perturbed residues in the basic cleft region of RPA70N. R43, S55, T60, Y118, and...
E120 were significantly changed more than two standard deviations above the average. As in the WRN titration, CSPs of the C-terminal residues (Y118 and E120) are likely due to allosteric changes in the structure. The amplitudes of the \( \Delta \delta_{\text{avg}} \) values were smaller upon addition of FANCJ1120–1211 than of WRN422–484, similar to the effects of the BLM peptides. Figure 4C shows the location of FANCJ interacting residues on the structure of RPA70N (PDB ID: 2B29). Once again, they were clustered within the basic cleft of RPA70N.

Table 1. \( K_d \) values of FITC-labeled WRN peptides interacting with RPA70N or RPA70A.

|     | \( K_d \) (\( \mu \)M) |
|-----|------------------------|
| WRN435–450 | 41.4 ± 3.3 | 65.2 ± 8.1 |
| WRN426–436 | 63.1 ± 5.4 | 95.8 ± 8.4 |
| WRN441–450 | 29.3 ± 1.2 | 86.9 ± 17.7 |
| WRN435–476 | 14.9 ± 5.8 |

Figure 3. (A) Sequences of WRN422–484 polypeptide and WRN peptides (WRN435–450, WRN426–436, WRN441–450, and WRN435–476) for FPA assays and NMR experiments. (B) FPA of WRN peptides upon addition of RPA70N. FPA curves for the WRN peptides are in black (WRN435–450), blue (WRN426–436), and red (WRN441–450). (C) FPA of FITC-labeled WRN peptides upon titration with RPA70A. The color scheme is the same as in panel B. (D) FPA curve for WRN435–476.
Binding affinity of FANCJ peptides for RPA70N and RPA70A. We found that the C-terminal region of FANCJ (FANCJ1120–1211) specifically interacts with RPA70N. In order to define the core peptide region for the RPA70 binding, we performed FPA assays of two FITC-labeled peptides, FANCJ1120–1133 and FANCJ1201–1213, with RPA70N and RPA70A. Supplementary Fig. S3 shows anisotropy changes of FITC-labeled FANCJ peptides with RPA70N. The $K_d$ of the RPA70N-FANCJ1120–1133 complex was determined to be $40.2 \pm 1.8 \mu M$, and the $K_d$ of the RPA70N-FANCJ1201–1213 complex was estimated as $107.4 \pm 4.3 \mu M$ (Table 2). This data shows that FANCJ1120–1133 has a higher binding affinity for RPA70N than FANCJ1201–1213. Both peptides showed weaker binding to RPA70A than to RPA70N (see Supplementary Fig. S3). FANCJ1120–1133 and FANCJ1201–1213 bind to RPA70A ~4.4-fold and ~2.9-fold weaker than to RPA70N, respectively (Table 2). Our results suggest that the FANCJ-RPA interaction is mainly achieved via FANCJ1120–1133 and RPA70N.

Mapping of the FANCJ1120–1133 peptide binding surface on RPA70N. In order to map the FANCJ1120–1133 binding surface on RPA70N, we performed a CSP analysis. Figure 5A shows the overlaid $^1H$-$^15N$ HSQC spectra of 15N-labeled RPA70N in the absence or presence of increasing molar ratios of FANCJ1120–1211. (B) Averaged chemical shift perturbations ($\Delta \delta_{avg}$) in RPA70N upon interaction with FANCJ1120–1211. (C) Residues significantly shifted by FANCJ1120–1211 are mapped onto the crystal structure of RPA70N (PDB ID: 2B29). Interpretation of the color scheme and dashed lines are as described in Fig. 2.

| Peptide     | $K_d$ (μM) RPA70N | $K_d$ (μM) RPA70A |
|-------------|------------------|-------------------|
| FANCJ1120–1133 | 40.2 ± 1.8       | 175.8 ± 4.7       |
| FANCJ1201–1213 | 107.4 ± 4.3      | 318.8 ± 28.9      |

Table 2. $K_d$ values of FITC-labeled FANCJ peptides interacting with RPA70N or RPA70A.

Y1131 and F1132 of FANCJ are critical residues for RPA70N binding. Previous experiments with an ATRIP-based unnatural peptide (DFTADDLEEWAL) showed that the aromatic residues in the C-terminus of the peptide improved its binding affinity for RPA70N[25]. FANCJ possesses two aromatic residues, Y1131 and F1132, at the end of the first acidic repeat. In order to investigate the effects of these aromatic residues on RPA70N binding.
binding by FANCJ, we prepared point mutants, Y1131A and F1132A, of FANCJ 1120–1133 (Fig. 6A). In an FPA competition assay with FITC-labeled FANCJ 1120–1133 and RPA70N, the fluorescence signal was not changed with increasing amounts of Y1131A and F1132A (Fig. 6B). This data showed that neither mutant could compete against the wild-type sequence for binding to RPA70N. We also performed 1H-15N HSQC experiments on 15N-labeled RPA70N titrated with both mutants. Figure 6C,D show the Δδ avg of RPA70N upon binding to Y1131A and F1132A, respectively. Strikingly, almost no significant chemical shift changes were observed, not only in the basic cleft region, but across the entire protein. Our FPA competition assay and CSP analysis show that both mutants have much lower affinities for RPA70N compared to the wild-type peptide. This suggests that both aromatic residues, Y1131 and F1132, at the C-terminal end of FANCJ1120–1133 are crucial for RPA70N binding.

Discussion

In this study, we investigated RPA’s interactions with peptides from human helicases, WRN and FANCJ, to identify core sequences for RPA binding. Our CSP analysis showed that WRN 122–484, which contains two full acidic repeats, specifically interacts with RPA70N via the basic cleft region. We also monitored significant CSPs in the basic cleft region of RPA70N upon binding to WRN 35–450, which contains only one acidic repeat. Even though the binding affinity of WRN 35–450 for RPA70N is weaker than previously reported cases of BLM peptides, WRN has two tandem RPA binding sites that can compensate for the lower affinity. At the same time, WRN 35–450 binds to RPA70A with a Kd of 65.2 ± 8.1 µM, as determined by FPA assay. This suggests that WRN 35–450 could interact with both RPA70N and RPA70A, which are connected with a flexible linker. Thus, the proximity of the two binding sites (WRN 35–450 and WRN 461–476) could enhance the overall binding affinity compared to that of the individual sequences. The low Kd value (14.9 ± 5.8 µM) of WRN 435–475 with RPA70N-A measured in this study strongly supports this hypothesis. Our data suggest that the WRN-RPA interaction is a multivalent binding, where RPA70N serves as the primary binding site with higher affinity and RPA70A is the secondary binding site. This is consistent with previous research showing several RPA binding partners interacting through multiple contact points, with one contact via RPA70N or RPA32C, and a secondary weaker contact within the RPA70AB domain.
Regarding RPA70A’s binding affinity for WRN435–450, it is higher than for Rad51, but both of them have much lower affinity compared to the ssDNA-RPA70A complex. This implies that the interaction may not occur with ssDNA-bound RPA. However, more investigations are necessary to reveal the complex interactions between WRN, RPA, and DNA substrates.

Even though the physical and functional interaction between FANCJ and RPA was reported, their binding surfaces were not revealed yet. We hypothesized that the acidic-rich sequence in the unstructured C-terminal region of FANCJ could be a candidate for RPA70N binding based on the unique RPA binding property of FANCJ among superfamily of 2 Fe-S helicases and the sequence conservation. Our NMR and FPA results clearly showed that one of the candidates, FANCJ1120–1133, could specifically interact with RPA70N via the basic cleft region. The binding had a dissociation constant of about 40 μM, which is similar to the WRN435–450-RPA70N interaction. This result suggests that FANCJ1120–1133 binds to RPA70N stronger than RAD9, MRE11, and p53, but weaker than BLM and ATRIP. We also found that two aromatic residues, Y1131 and F1132, in the C-terminus of FANCJ1120–1133 are critical for RPA70N binding. This suggests that the FANCJ-RPA interaction is not only electrostatic but also hydrophobically tuned, consistent with the results for FANCJ1201–1213, which does not have aromatic residues at the C-terminal end and has a very low affinity for RPA70N.

It has recently been recognized that weak and transient protein-protein interactions, with a Kd in the micro-molar to millimolar range, are important for the cell's signaling, regulatory, and stress response mechanisms. Accordingly, RPA-mediated protein-protein interactions should not be too strong or persistent, because RPA must interact with the appropriate partner depending on the cellular conditions, such as DNA damage response or repair processes. In this context, the modest binding affinities of RPA-WRN and RPA-FANCJ could be physiologically relevant.
Supplementary Fig. S4 shows the sequence comparison of the RPA70N binding regions of BLM, ETA1, ATRIP, WRN, and FANCJ. The acidic-rich region in the middle combined with the distribution of hydrophobic residues is known to be crucial for RPA binding. Both FANCJ1120–1133, and WRN437–449 share these common features. We also compared the binding surfaces on RPA70N for each acidic peptide from WRN, FANCJ, and BLM18 (see Supplementary Fig. S4). While residues near 555 participate in the binding of all three proteins, the T60 site does not appear to make contact with the WRN and FANCJ peptides. Thus, the BLM peptides make contact over a relatively wider area. This could be related to the fact that BLM peptides have lower Kd values. We also performed docking simulations for WRN441–456–RPA70N and FANCJ1120–1133–RPA70N on the CABS-Dock Webserver30 (see Supplementary Tables S1 and S2). Supplementary Fig. S4 shows the representative model of each complex. Both peptides are located in the basic cleft region between two loops.

While WRN binds to RPA in its N-terminal region, RPA binding region of FANCJ (FANCJ1120–1133) is located at the C-terminal region of the protein. A previous report showed that the spatial position of RPA70N is important for optimal stimulation of WRN's helicase activity from the 3' to 5' direction31. The relative orientations of the helicases to RPA may depend on the location of the binding regions. We hypothesize that the opposite directionality of FANCJ's helicase activity compared to BLM and WRN may be related to RPA's binding in its C-terminus.

In summary, we investigated whether acidic peptides of WRN and FANCJ bind to RPA70N or RPA70A through NMR spectroscopy and FPA assays. Peptides of both proteins bound to RPA70N with Kd's in the micromolar range, and we identified FANCJ1120–1133 as a novel RPA70N binding site. Tandem acidic repeats of WRN mediate multi-domain binding. Our study provides valuable information on RPA interactions with WRN and FANCJ helicases, which may be useful for developing therapeutic strategies for cancer treatment.

Methods
Sample preparation. We used five fluorescein isothiocyanate (FITC)-labeled peptides (WRN435–456, WRN426–456, WRN441–456, FANCJ1120–1133, and FANCJ1201–1213). All were purchased from AnyGen (Gwangju, Korea). These peptides were purified using HPLC with acetonitrile containing 0.05% TFA (trifluoroacetic acid) to 95% purity. Three unlabeled peptides (WRN435–456, FANCJ1120–1133, and FANCJ1201–1213) were used for NMR experiments. These were also purchased from AnyGen and purified with the same method.

RPA70N and RPA70A and a tandem construct comprising RPA70N followed by RPA70A with (GGGGG)3 linker were subcloned into a PET15b vector and transfected into BL21 (DE3) cells. The proteins were overexpressed and purified as described previously16,32. 15N-labeled proteins were obtained by growing cells in M9 medium containing 15NH4Cl and unlabeled D-glucose. FANCJ1120–1211, WRN422–484, and WRN435–475 were subcloned into a pET His6 TEV LIC cloning vector (2B-T) (a gift from Scott Gradia, Addgene plasmid #29666) and then transfected into BL21 (DE3) and Rosetta (DE3) cells, respectively. The cells were grown at 37°C to an OD600 of 0.5–0.6, at which time IPTG was added to a final concentration of 1.0 mM. Cells were incubated for an additional 20 h at 18°C. His-tagged WRN and FANCJ proteins were purified using a Ni-NTA column (Elpis Biotech, Korea) and eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 300 mM imidazole). All proteins were loaded onto a Superdex 75-pg FPLC column (GE Healthcare) pre-equilibrated with 20 mM HEPES (pH 7.4), 100 mM NaCl, and 1 mM DTT for further purification.

NMR experiments. 1H-15N HSQC experiments were performed using a Bruker 900 MHz NMR spectrometer equipped with a cryogenic probe (KBSI, Ochang). We used Bruker basic pulse sequence ‘hsqctf3gps2’. The detailed parameters are as follows: Size of Free Induction Decay (TD), 2k (1H dimension)/192 (15N dimension); Size of real spectrum (SI), 2k/1k. 15N-labeled RPA70N and RPA70A were dissolved in 20 mM HEPES (pH 7.4), 100 mM NaCl, and 1 mM DTT at 0.3 mM. For WRN and FANCJ titrations, WRN422–484 and FANCJ1120–1213 were added at molar ratios of 0, 0.5, 1.0, and 2.0, and WRN435–456, FANCJ1120–1133, and FANCJ1201–1213 were added at molar ratios of 0, 0.1, 0.5, 0.75, 1.0, and 2.0. All experiments were performed at 298 K. Published amide chemical shifts of RPA70N and RPA70A were used to analyze C53,54. Topspin was used to process the NMR spectra, and data analyses were performed with Sparky35. The average chemical shift changes (Δδavg) were calculated according to equation [1],

$$\Delta \delta_{avg} = \sqrt{(\Delta \delta_{H})^2 + (\Delta \delta_{N}/5.88)^2}$$

(1)

where ΔδH and ΔδN are the amide proton and nitrogen resonance chemical shift changes, respectively. Residues with changes greater than one or two standard deviations from the average CSP were considered to be significantly perturbed.

Fluorescence polarization anisotropy experiments. A FITC label with a 6-aminohexanoic acid spacer at the N-terminus of WRN and FANCJ peptides was used for the FPA assays. The fact that FITC does not have a substantial effect on the interaction between RPA70 and the peptides was confirmed by an experiment comparing the affinity of FITC-labeled and unlabeled ATRIP peptide, which is known to bind with RPA70N26. Because several acidic peptides for RPA binding have been tested with FITC labeling16,18,25, we used the same fluorescent probe for our assays.

Increasing concentrations (0–300 μM) of RPA70N (or RPA70A) in a total 50 μL of assay buffer (20 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.4) and 50 nM FITC-labeled peptides were mixed in Corning 96-well black plates (polystyrene, non-treated, flat bottom) and equilibrated for ≥1 h at 25°C. NHS-Fluorescein (Thermo Fisher, USA) conjugated isothiocyanate (FITC)-labeled protein (WRN435–456) and increasing concentrations of RPA70N-A (0–200 μM) were prepared in the same manner. Samples were excited at a wavelength of 485 nm and emission was detected at a wavelength of 528 nm using Cytation5 (BioTek) and Gene5 software (GIST, Gwangju). The emission polarization anisotropy was calculated as described in a previous report18. Data analyses were...
performed using GraphPad Prism version 7.01 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). At increasing concentrations of RPA70N or RPA70A, anisotropy curves were plotted. All experiments were repeated three times, and each dissociation constant (K_d) and its standard error was calculated from the 'one-site specific' fitting model. To compare the binding affinity of RPA70N and FANCJ1120–1133 with that of the FANCJ mutants (Y1131A and F1132A), an FPA competition assay was performed. To 6µM RPA70N and 500 nM FITC–FANCJ1120–1133 in 200µM assay buffer, 0–500µM Y1131A or F1132A were added and equilibrated as described above. The 'log(1+Inhibitor) vs. response – variable slope (four parameters)' method of the GraphPad software was applied to the plot. The equation used for the fitting is as follows:

\[ Y = Bottom + \frac{Top - Bottom}{1 + 10^{\left(\log IC_{50} - X\right)/HillSlope}} \]

Top and Bottom values are plateaus in the units of the Y axis. IC_{50} is the inhibitor concentration that generates a response half way between Bottom and Top. HillSlope represents the steepness of the curve.

However, the fitting was not possible because unlabeled mutant peptides failed to compete with the labeled one (wild-type).

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Author Contributions
G.Y., J.K. and C.-J.P. designed the study. G.Y. and J.K. prepared samples. G.Y., J.K. and C.-J.P. planned and performed experiments. G.Y., J.K., and C.-J.P. analyzed the data. G.Y., J.K., and C.-J. P wrote the paper.

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