Crystal Structure of the HRDC Domain of Human Werner Syndrome Protein, WRN*

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Werner syndrome is a human premature aging disorder characterized by chromosomal instability. The disease is caused by the functional loss of WRN, a member of the RecQ-helicase family that plays an important role in DNA metabolic pathways. WRN contains four structurally folded domains comprising an exonuclease, a helicase, a winged-helix, and a helicase-and-ribonuclease D/C-terminal (HRDC) domain. In contrast to the accumulated knowledge pertaining to the biochemical functions of the three N-terminal domains, the function of C-terminal HRDC remains unknown. In this study, the crystal structure of the human WRN HRDC domain has been determined. The domain forms a bundle of α-helices similar to those of Saccharomyces cerevisiae Sgs1 and Escherichia coli RecQ. Surprisingly, the extra ten residues at each of the N and C termini of the domain were found to participate in the domain architecture by forming an extended portion of the first helix α1, and a novel looping motif that traverses straight along the domain surface, respectively. The motifs combine to increase the domain surface of WRN HRDC, which is larger than that of Sgs1 and E. coli. In WRN HRDC, neither of the proposed DNA-binding surfaces in Sgs1 or E. coli is conserved, and the domain was shown to lack DNA-binding ability in vitro. Moreover, the domain was shown to be thermostable and resistant to protease digestion, implying independent domain evolution in WRN. Coupled with the unique long linker region in WRN, the WRN HRDC may be adapted to play a distinct function in WRN that involves protein-protein interactions.

Werner syndrome is a human premature aging disorder that results in premature aging. Werner syndrome gives rise to multiple progeroid pathologies, including osteoporosis, atherosclerosis, type II diabetes, cataracts, and a marked increase in the incidence of cancer (1, 2). Cells isolated from patients also display features of accelerated aging. In particular, genomic instability is characteristic of Werner syndrome cells, which show extensive chromosomal deletions, elevated rates of homologous recombination, prolonged S-phase DNA synthesis, and defective telomere maintenance (3–6).

Werner syndrome is caused by the loss of function of a single gene that encodes a 1432-residue protein, Werner syndrome protein (WRN)³. WRN is a member of the RecQ-helicase family that is widely distributed across the three domains of life (4–9). Whereas genomes of bacteria and unicellular eukaryotes typically encode a single recQ gene, the human genome contains five recQ genes, RecQ1, Bloom syndrome protein (BLM), WRN, RecQ4, and RecQ5. Mutations in BLM and RecQ4 cause Bloom syndrome and Rothmund-Thomson syndrome, respectively, and share a predisposition to cancer with Werner syndrome (9). Recently, the epigenetic inactivation of WRN in human cancer was demonstrated, illuminating the tumor-suppressor functions of WRN (10). Although little is known about the in vivo molecular pathways in which WRN is involved, its multiple interactions with key proteins in DNA replication, repair, recombination, and telomere maintenance suggest a coordinated function of WRN with these processes in human cells (4–6, 9, 11).

WRN contains four structurally folded domains (see Fig. 1) comprising an exonuclease (12), a helicase, a winged-helix (13), and a helicase-and-ribonuclease D/C-terminal (HRDC) domain. Most mutations found in Werner patients occur in the C-terminal region of the protein, resulting in C-terminal truncated proteins with loss of the nuclear localization signal (NLS) (14). The helicase and winged-helix domains combine to form the catalytic core of the protein. Detailed studies of core fragments from Escherichia coli RecQ (15, 16) and BLM (17) demonstrated that the fragment is sufficient for ATPase and DNA unwinding activities in vitro, whereas the function of the C-terminal HRDC domain has not been elucidated. As its name implies, this region shares sequence homology with a domain in the RNase D family of nucleases (18). The HRDC domains of Saccharomyces cerevisiae Sgs1 (yeast homologue of RecQ proteins) (19) and E. coli RecQ (20) have been suggested to function as auxiliary DNA binding domains. HRDC is the most vari-

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1 The atomic coordinates and structure factors (code 2E1E and 2E1F) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: WRN, Werner syndrome protein; BLM, Bloom syndrome protein; HRDC, helicase-and-ribonuclease D/C-terminal; DSB, double-strand breaks; GST, glutathione S-transferase; DTT, dithiothreitol; CAPSO, 3-(3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonic acid; MR, molecular replacement; TOF-MS, time-of-flight mass spectrometry.
able of the RecQ family domains (WRN HRDC region shares only ~20% homology with Sgs1 and E. coli RecQ) with examples of human proteins that lack the HRDC domain altogether, including RecQ1, RecQ4, and all isoforms of RecQ5. The function of the HRDC domains in WRN and BLM might be more diverse than as represented in the proteins of unicellular organisms. In addition, WRN is distinct from E. coli RecQ in having a considerably long linker region (>70 residues) that connects HRDC to the N-terminal winged-helix domain. An interesting point concerns how the isolation of the HRDC domain contributes to the specific functions of WRN.

In an effort to delineate the biochemical function of HRDC in WRN, we have determined the crystal structure of the human WRN HRDC domain. This represents the first structure of a mammalian RecQ family HRDC domain. The structure demonstrated that a bundle of α-helices forms the domain core similar to those of Sgs1 (19) and E. coli RecQ (20). Surprisingly, the additional ten residues at each of the N and C termini, which are disordered or truncated in Sgs1 and E. coli, were found to form a novel motif on top of the WRN HRDC domain. The domain was further shown to be stable in solution and lack DNA binding ability.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of WRN HRDC Domain**—DNA encoding residues 1142–1242 of WRN was cloned from human E. coli strain BL21-CodonPlus RIL (Stratagene), which was grown in LB media supplemented with 50 μg/ml ampicillin at 310 K to an 660 of 0.5. Expression was induced by the addition of isopropropyl-β-D-thiogalactoside to 0.5 mM. Following incubation for 27 h, cells were pelleted by centrifugation and washed 2 times with 0.9% (w/v) NaCl solution. The cell suspension was clarified by ultracentrifugation at 37,000 rpm for 30 min. The supernatant, which contains GST-WRN HRDC, was loaded onto a glutathione-Sepharose-4B affinity column (Amersham Biosciences) to remove nucleic acids. The flow-through fraction was pooled and concentrated by centrifugation using an Amicon Ultra 5000 MWCO. The protein was finally loaded onto a HiLoad 16/60 Superdex 75 gel-filtration column (Amersham Biosciences) equilibrated with a buffer comprising 50 mM Tris- HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT (buffer A). The column was washed with buffer A until protein was undetectable in the eluent and subsequently re-equilibrated with digestion buffer comprising 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM CaCl2, and 1 mM DTT. The protein bound to the resin was digested on the column with 10 unit/ml Factor Xa (Novagen) for 12 h at 293 K. The column effluent containing GST-free HRDC was pooled and then loaded onto a HiTrap Q anion-exchange column (Amersham Biosciences) to remove nucleic acids. The flow-through fraction was pooled and concentrated by centrifugation using an Amicon Ultra 5000 MWCO. The protein was finally loaded onto a HiLoad 16/60 Superdex 75 gel-filtration column (Amersham Biosciences) equilibrated with buffer A. The peak fractions, which gave one major band corresponding to ~10 kDa in SDS-PAGE analysis, were pooled and concentrated to ~50 mg/ml in buffer A. Ten milligrams of purified HRDC was obtained from 1 liter of culture. The sample was analyzed by N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (Bruker), which verified that the protein had been successfully purified without degradation. Two vector-derived residues (Gly-Ile) were present at the N terminus. The sample was stored at 193 K until use for crystallization.  

**Crystallization**—Crystallization screening was carried out by the vapor-diffusion method at 20 °C using commercial screening kits (Hampton Research and Nextal Biotechnologies). The protein was mixed in a 1:1 ratio with the reservoir solution. The crystallization conditions were subsequently optimized by the sparse matrix method. The prismatic crystal (space group I4122) was obtained under conditions that included 1.6–2.2 M ammonium sulfate, 5% (v/v) polyethylene glycol (PEG) 400, and 0.1 M CAPSO-NaOH (pH 9.5). The same I4122 crystal also grew by the same method with 0.9% (w/v) PEG4000 or 20–28% (w/v) PEG4K as a precipitant. Irrespective of the precipitants, the crystal appeared within a wide range of pH (from 5 to 10). In contrast, the hexagonal crystal (space group P6122) was obtained within a limited pH (~5.5), under conditions that included 30–40% (v/v) PEG400, 0.1 M NaCl, and 0.1 M citrate-NaOH (pH 5.5). These conditions partially overlap with those for the I4122 crystal, and in fact the two forms of crystals often grew concomitantly from the same crystallization drop.  

**X-ray Data Collection**—For x-ray data collection, the I4122 crystal obtained with use of ammonium sulfate was transferred into a cryoprotective solution containing 15% (v/v) glycerol in the reservoir solution. The crystal was mounted on a rayon loop and flash-frozen in liquid nitrogen. The x-ray data were collected at SPring-8 on beamline BL38B1. The crystal was maintained at 100 K in a nitrogen stream during exposure to the x-ray beam with a wavelength of 1.00 Å. Diffraction from a total oscillation range of 180° was recorded by the charge-coupled device detector (Rigaku MSC Jupiter 210). The data set was indexed and merged using the HKL2000 program suite (21) to a resolution limit of 2.3 Å. The other two P6122 crystals obtained from use of PEG400 and PEG4K also possessed similar diffraction qualities (resolution limits of 2.6 and 2.4 Å, respectively). The P6122 crystal was directly frozen in liquid nitrogen without cryoprotection, and the x-ray data were collected to a resolution limit of 2.0 Å.  

**Structure Determination**—Given the crystallization difficulties encountered for the P6122 crystal, x-ray data were initially only obtained from the I4122 crystal. The phase-determination trials by the molecular replacement (MR) method were performed against the I4122 data, using the HRDC structures of S. cerevisiae Sgs1 (19) and E. coli RecQ (20) as an MR model. The MR search using either of the models was unsuccessful, probably due to the lack of homology with human WRN. Following this, a calculated MR model was constructed using the homology-modeling server SWISSMODEL (22), to which the amino acid sequence of WRN and both of the two template structures (Sgs1 and E. coli HRDCs) were submitted. The model obtained was further converted to an alanine model. Using this calculated structure as a new MR model, PHASER (23) successfully gave a solution corresponding to one HRDC
molecule in the asymmetric unit of the crystal. The electron-density map obtained was of good quality and allowed for further model building of the WRN HRDC domain. The extreme N- and C-terminal residues, 1142–1151 and 1227–1235, respectively, that are missing in the search model were modeled de novo using program O (24), and the structure was refined using CNS (25) and REFMAC with TLS parameterization (26). Subsequently, using this refined I4₂/₂₂ structure as a search model, the phases for the P6₂/₂₂ data were determined by MR, followed by refinement using the same programs. The figures were prepared using PyMOL (DeLano Scientific, San Carlos, CA).

Ultracentrifuge Sedimentation Equilibrium—Purified WRN HRDC was dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1 mM DTT followed by adjustment of the absorbance at 280 nm to ~0.5 (corresponding to a protein concentration of ~300 μM). The sample solution and reference buffer were injected into each side of a double-sector centerpiece. Ultracentrifugation was performed at 4 and 20 °C at 25,000 rpm using a CP100α unit provided by Hitachi High-Technologies, Japan. Following equilibration (50–60 h later), the radial absorbance at 280 nm was scanned, which subsequently fitted to the calculated curve from the ideal single-species model. The partial specific volume of the protein at 20 °C was calculated to be 0.745 ml/g based on the amino acid composition, whereas the solvent density was calculated to be 1.0049 g/ml (4 °C) and 1.0031 (20 °C).

Dynamic Light Scattering—Dynamic light scattering of WRN HRDC was measured using the DynaPro-801 equipped with DYNAMICS software (Protein Solutions). The sample was prepared at 3.8 mg/ml protein concentration (corresponding to ~300 μM) in buffer A and filtered using a 0.02-μm membrane. Measurements were performed at temperatures ranging from 5 to 55 °C, with increments of 5 °C. Each measurement was repeated 50 times and averaged.

Limited Proteolysis—WRN 1142–1242 in GST-tagged and non-tagged forms were purified and prepared at 10 mg/ml in buffer A. Elastase from porcine pancreas (Wako Pure Chemical Industries, Japan) was dissolved to 1 mg/ml in the same buffer, which was subsequently added to the samples at increasing concentration from 0 to 300 μg/ml. Following incubation at room temperature for 1 h, the digested samples were analyzed by SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane for N-terminal sequencing. The products were also analyzed by TOF-MS to define the correct domain boundaries of WRN HRDC.

Fluorescence Polarization Assays—Oligonucleotides representing various DNA structures were designed based on the study by Mohaghegh et al. (27). In each substrate, a single oligonucleotide was 3′-end-labeled with fluorescein isothiocyanate (Hokkaido System Science, Japan). The labeled oligonucleotide was annealed to the unlabeled complementary strand(s) at a 1:1 molar ratio by incubation at 90 °C for 3 min followed by slow cooling to room temperature. Purified WRN HRDC was mixed with each substrate in a solution containing 10 mM Tris-HCl (pH 7.5), 25 mM NaCl, and 1 mM DTT. The final protein concentration ranged from 1 nM to 50 μM, whereas the DNA substrate concentration was 100 pM in all samples. The interaction between protein and DNA was examined by the fluorescence polarization method (28) using the full-range Beacon 2000 (Invitrogen). Each measurement was performed at 25 °C, repeated ten times, and then averaged. As a positive control, the WRN winged-helix domain comprising residues 949–1079 was purified and used.

RESULTS

Domain Characterization and Crystallization—To date, the purification and structure determination of RecQ family HRDC domains has only been reported for two unicellular proteins, S. cerevisiae Sgs1 (19) and E. coli RecQ (20), and there have been no reports on the successful isolation and purification of the HRDC domains from mammalian RecQ proteins. From the sequence alignment with Sgs1 and E. coli RecQ, the literature data limit the WRN HRDC region to residues 1152–1226 (Fig. 1). However, this lacks accuracy given that the primary sequences of the three proteins are significantly divergent.

Limited proteolysis of WRN using subtilisin suggested that the HRDC domain may start at Gln1148 (29), whereas no information was available about the C-terminal end of the domain. At first we performed sequence analysis of WRN using the domain linker prediction server (30). The program suggested significant flexibility in residues 1132–1147 and 1230–1246 that are at the N and C termini, respectively, of the predicted HRDC region. At the same time, from the sequence alignment of human WRN with mouse and Xenopus WRNs (supplemental Fig. S1), residues 1142–1242 that include the HRDC region were found to represent a conserved cluster region. Taken together, a recombinant WRN HRDC fragment comprising residues 1142–1242 was constructed, which satisfies all of the aforementioned arguments.

The HRDC fragment was overexpressed in E. coli as a GST-tagged protein and finally purified in a tag-cleaved form to homogeneity. CD spectral analysis of the protein (supplemental Fig. S2) indicated that the fragment contains a high amount of α-helical content (50%) and a low amount of β-strand content (6%). The helical content is similar to that of Sgs1 and E. coli RecQ structures (19, 20), suggesting that the purified fragment folds into a similar domain architecture. Crystallization conditions were screened by the vapor-diffusion method, from which a number of small crystals appeared under conditions that included ammonium sulfate or PEG as a precipitant. Following the refinement of conditions and preliminary x-ray analysis, it was found that the protein crystallizes in two different crystal forms, space groups I4₂/₂₂ and P6₂/₂₂ (supplemental Fig. S3, A and B, respectively). X-ray data sets were collected from the crystals to a resolution limit of 2.3 (I4₂/₂₂) and 2.0 Å (P6₂/₂₂). For the structure determination, phases were obtained by the MR method using S. cerevisiae Sgs1 (19) and E. coli RecQ (20) structures as a search model (see “Experimental Procedures”). Crystallographic statistics are summarized in Table 1.

Overall Structure—In the electron density maps calculated from the I4₂/₂₂ and P6₂/₂₂ data, the protein density is clear throughout the sequence except for the C-terminal seven residues (1236–1242) being disordered. The two structures, both of which include a model for 1142–1235, are essentially the

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same when superimposed with a root mean square deviation of 0.8 Å (94 Ca atoms, supplemental Fig. S3C). Both of the crystals contained one HRDC monomer in the asymmetric unit. The solvent content of the I4,22 crystal was considerably high (71.6% of the total volume, cf. 62.7% in the P6,22 crystal), which resulted in less contact area between the packed molecules. The following discussion concerns the I4,22 structure.

The structure revealed that residues 1152–1226 of the WRN fragment fold into a conventional HRDC domain composed of five α-helices and one 310-helix packed together (Fig. 2A). The helices are connected by loops that are well defined in the electron-density map. Many of the hydrophobic residues assemble to form a domain core, in which Leu1160, Leu 1184, Met 1187, and Ile1219 are especially important for hydrophobic interactions. The four residues are highly conserved in WRN, Sgs1, and E. coli RecQ, as well as hydrophobic in BLM (Fig. 2B).

Within the range of this conventional HRDC domain (residues 1152–1226), the structure was superimposed on those of Sgs1 (19) and E. coli RecQ (20) with root mean square deviations of 2.4 and 2.0 Å, respectively (74 Ca atoms, Fig. 2B).

Surprisingly, the additional 10 residues at each of the N and C termini of the domain core, which are located beyond the conventional HRDC region and are disordered (or lacking) in Sgs1 and E. coli, were found to form a novel motif unique to WRN.

**FIGURE 1.** Amino acid sequence of human WRN HRDC. Domain diagram of full-length WRN is shown on top, whereas the amino acid sequence of HRDC is shown below. Sequences of S. cerevisiae Sgs1 and E. coli RecQ are aligned based on their three-dimensional structures (19, 20). Sequence of human BLM, the structure of which is still unknown, is also aligned for comparison. Secondary-structure elements (helices) are shown below each sequence as green (WRN, present work), pink (Sgs1), and yellow (E. coli) bars, respectively. Loop regions are shown as a solid line, while the disordered seven residues at the C terminus of WRN HRDC are shown as a dashed line. The N- and C-terminal additional residues in WRN that participate in HRDC architecture are highlighted in red (all of these comprise the hydrophobic core of the domain).

**TABLE 1**

Crystallographic data for the WRN HRDC domain

| X-ray data | I4,22 | P6,22 |
|-----------|-------|-------|
| Space group | I4,22 | P6,22 |
| Cell parameters (Å) | 64.46, c = 189.71 | 61.66, c = 136.68 |
| Resolution (Å) | 2.30 | 2.00 |
| Mosaicity | 0.2–0.3 | 0.3–0.5 |
| Reflections, total/unique | 115,494/9,021 | 216,976/10,934 |
| Completeness (%) | 96.8 (77.6) | 99.7 (99.6) |
| Rmerge (%) | 18.3 (8.0) | 20.6 (9.8) |
| Rwork/Refinement (%) | 4.33/71.6 | 4.89/69.0 |

**Refinement**

| | I4,22 | P6,22 |
| Protein | 94 (of 101) | 94 (of 101) |
| Solvent | 30 | 53 |
| Ramachandran plot (%) | 25.3/27.9 | 24.8/25.5 |
| Most favored | 90.5 | 91.7 |
| Additional allowed | 9.5 | 8.3 |
| Generously allowed | 0 | 0 |
| Disordered | 0 | 0 |
| Average B-factor (Å²) | 0.010, 1.1 | 0.010, 1.0 |

* Numbers in parentheses refer to statistics for the outer resolution shell.

* Rwork = Σ|Fcalc| - |Fc||ΣFcalc|, Rfree is the same as Rwork except that a 5% subset of all reflections was held aside throughout the refinement.
Structures of Human WRN HRDC Domain

**Figure 2. Crystal structure of the human WRN HRDC domain.** A, front view (left) and top view (right) of WRN HRDC in a ribbon model. Secondary structure elements are labeled, and dimensions of the molecule are indicated. Molecular surface of the domain is shown in transparent gray (conventional HRDC core) and red (N- and C-terminal extensions unique to WRN). B, superimposition of WRN HRDC (green) with Sgs1 (19) (pink) and *E. coli* (20) (yellow) HRDCs. The orientation is the same as in A. The N and C termini of each molecule are labeled. Superimposition was performed using LSQMAN (56).

All of the six side chains from Val^{1228}–Phe^{1233} in the loop are included in the interaction, which together contribute to the binding specificity. Among the interactions, hydrophobic interactions are especially important. The groove of the HRDC core includes two hydrophobic sites, pocket-1 and pocket-2 (Fig. 3B), within which the hydrophobic side chains of Val^{1228} and Phe^{1233}, respectively, are buried deeply. The hydrophobicity of these two residues is also conserved in mouse and *Xenopus*, but not in Sgs1 or BLM (Fig. 1 and supplemental Fig. S1). At the same time, a total of seven hydrogen bonds are formed between the loop and the domain core within a distance of 2.9 Å (Fig. 3B).

On the other hand, the N-terminal elongated portion of helix α1 protrudes from the HRDC core in the proximity of the C-terminal extended loop (Fig. 2A). Interestingly, the C-terminal loop and helix α1 protrusion stabilize each other’s conformation. The methyl group in the side chain of Thr^{1152} on α1 participates in formation of pocket-1, thus interacting with the side chain of Val^{1228} on the C-terminal loop. Furthermore, Glu^{1149} on α1 stabilizes the extended side-chain conformation of Arg^{1191} through ionic interactions, which contribute to the formation of three hydrogen bonds between Arg^{1191} and main chain atoms of the C-terminal extended loop.
WRN HRDC Is a Monomer in Solution—In the purification of WRN HRDC using gel-filtration chromatography, we noticed that the protein eluted at a position close to the 25 kDa molecular mass marker (data not shown). The WRN samples at concentrations ranging from 60 to 600 μM were examined, all of which gave a single peak at the same elution volume. From the calibration curve the molecular mass was estimated to be 20 kDa, which is almost twice as large as the calculated mass of the monomer (11.4 kDa). In contrast to E. coli RecQ, which functions as a monomer (31), full-length WRN has been suggested to form multimers (32). An interesting point concerns whether the isolated HRDC domain can undergo oligomerization. Although the gel-filtration data suggests dimerization of HRDC in a concentration-independent manner, the method employed is often affected by the molecular shape and surface property of the protein sample.

Ultracentrifugation was employed in an effort to define the assembly state of the WRN HRDC domain. WRN HRDC at 300 μM was analyzed by the sedimentation-equilibrium method at 20 °C. The resultant absorbance data (Fig. 4A) fitted well to the calculated curve from the ideal single species model. The molecular mass was calculated to be 10.7 kDa, which agrees well with the values of the monomer. A similar result (11.2 kDa) was also obtained at 4 °C.

Furthermore, the protein was analyzed by the dynamic light scattering method. The experiment was performed using a sample at the same protein concentration as in the ultracentrifugation (300 μM), while the temperature was elevated stepwise from 5 to 55 °C, with increments of 5 °C. The result, shown in Fig. 4B, demonstrated that the protein is highly monodisperse in solution with a hydrodynamic radius of ~20 Å. This radius is closer to the value determined for the monomer structure (Fig. 2A) than the several dimer structures generated from crystallographic symmetry (data not shown). Consequently, it was concluded that WRN HRDC would exist as a monomer in solution. The slightly early elution in the gel filtration might be due to the unique domain architecture of WRN HRDC, where the height (~42 Å) is significantly larger than the width and depth (~26 Å, Fig. 2A).

WRN HRDC Is a Highly Stable Domain—In the dynamic light scattering (Fig. 4B), it was unexpectedly revealed that WRN HRDC is highly thermostable, at least up to 55 °C (limit of the instrument), without any significant change in the hydrody-
The protein sample used in the experiment was subsequently examined using an optical microscope, which confirmed that the protein remained soluble with no aggregation.

Limited proteolysis of WRN HRDC was performed to demonstrate that the domain extension by terminal residues (elongated helix α1 and C-terminal extended loop) did not represent a crystallization artifact but maintained in solution. Purified WRN HRDC (residues 1142–1242) in both GST-tagged and non-tagged forms were treated with increasing amounts of elastase ranging from 0 to 300 μg/ml (Fig. 4C). TOF-MS and N-terminal sequencing then delineated the degradation products. The rationale behind this approach is that unfolded residues are generally less resistant to proteases than folded residues. The GST tag serves as an internal control of a protease-resistant folded domain, which began being degraded at 50 μg/ml elastase (lane 3) and at 300 μg/ml was mostly degraded (lane 5). In contrast, WRN HRDC was shown to be extremely stable even at the highest concentration of protease (300 μg/ml, lanes 5 and 10). Both the GST-tagged and non-tagged forms yielded the same WRN fragment comprising residues 1145–1242, where digestion occurs only in the N-terminal three residues (I1142QPV/I1144) of the fragment, whereas no cleavage is observed in the C-terminal residues. The result consistently indicates that both the N- and C-terminal extensions fold into a protease-resistant structure in solution, as shown by the two crystal structures (I4122 and P6222) that form different packing interactions.

It should be added that the crystals of WRN HRDC (I4122 crystal) grew in a wide range of pH (from 5 to 10), indicating that the domain is stable against low and high pH. At somewhat neutral pH (7.5), the sample can be concentrated to at least 80 mg/ml with no aggregation. In summary, the WRN HRDC domain is a highly stable and soluble domain that is resistant to high temperature, low and high pH, and protease digestion.

**Surface Property of WRN HRDC**—The electrostatic surface potential of WRN HRDC is shown in Fig. 5A. The front surface of the domain is roughly negatively charged and punctuated with neutral and basic amino acids (left, front view), whereas the back surface is generally positively charged and includes hydrophobic residues (right, back view).

To date, the DNA binding activity of RecQ family HRDC domains has been intensively studied for Sgs1 and *E. coli* RecQ. NMR chemical shift assays of Sgs1 HRDC identified eight residues (mapped in Fig. 5B) as a determinant for interaction with single-stranded DNA and partially double-stranded DNA with a single-stranded overhang (3’-overhang duplex) (19).
residues form a positively charged and partially hydrophobic region on the front surface of Sgs1. The region may recognize phosphate backbones of DNA, although the interaction is markedly weak (dissociation constant around $30 \mu M$) (19). The present study has revealed that WRN HRDC also possesses a positively charged region that includes Asn1169 and Lys1170 (corresponding to Sgs1 residues Asn1291 and Arg1292, respectively) at the bottom right of the front surface (Fig. 5A, front view). However, the other basic residues are not conserved, with Lys1329 and Arg1333 in Sgs1 replaced by nonpolar Gly1207 and Met1211, respectively, in WRN, whereas Ala1339 is replaced by acidic Glu1217. Consequently, the charge distribution of WRN at the bottom left of the front surface is markedly changed and comprises negatively charged and uncharged residues, which may exclude any interaction with DNA.

On the other hand, electrophoretic mobility shift assays of E. coli RecQ HRDC demonstrated that the domain also binds to single-stranded DNA and 3'-overhang duplex with a dissociation constant of $2.5 \mu M$ (20). Mutation assays further demonstrated that E. coli uses a different face of the domain when binding to DNA, which includes five basic residues and one aromatic residue (mapped in Fig. 5B). In WRN, the two basic residues Arg1164 and Lys1208 (corresponding to E. coli Arg543 and Lys587, respectively) are also conserved, preserving the lower right portion of the positively charged back surface (Fig. 5A, back view). However, the other three basic residues, including E. coli Lys534, Arg540, and Lys544, are replaced by hydrophobic residues Val1155 and Val1161, and the bipolar residue Gln1165, respectively, markedly changing the left side of the E. coli site to one that is hydrophobic and negatively charged in WRN. This area is also stacked by a negatively charged cluster provided by the extended portion of helix 1.

Moreover, E. coli residue Tyr555 located on the $3_{10}$-helix is substituted by Ala1176 in WRN. As described above, the tyrosine side chain is especially important for E. coli in interacting with DNA, where replacement with alanine dramatically disrupts the binding (20).

As a consequence of the additional terminal residues stacked on top of the HRDC core, the following two unique surface properties appear in WRN. Firstly, the binding of the C-terminal extended loop that includes Asp1231 generates a large acidic/hydrophobic patch at the head portion of the domain (boxed...
by dashed line in Fig. 5A, front view). The region is unique to WRN and is lacking in Sgs1 or E. coli. Secondly, the N-terminal extension of helix α1 provides a hydrophobic pocket on the opposite side of the domain (Fig. 5A, back view, magnified in Fig. 5C). The pocket is surrounded by hydrophobic atoms from six residues comprising Gln1150, Gln1153, Ile1154, and Tyr1157 at the N-terminal half of α1, and Val1185 and Lys1189 on helix α2. In the electron-density maps both from I4_22 and P6_22 crystals, a strong and large residual density with ellipsoid shape was observed that buried this pocket. The density possesses a diameter of ~2–3 Å, which seems to stack on the aromatic ring of Tyr1157 with a distance of ~4 Å. Tyr1157 is conserved among human, mouse, and Xenopus WRNs. Judging from the hydrophobic environment it binds, the density would represent some nonpolar atoms of a small molecule included in the crystallization. A possible candidate is Tris (buffer) or glycerol (cryoprotectant), although its identification was unsuccessful. The density appears only in a round shape probably due to disorder and/or multiple conformations. Currently no model has been built into this density.

**WRN HRDC Is Defective in DNA Binding**—To assess the DNA binding ability of WRN HRDC in solution, we employed a fluorescence polarization assay (28) using fluorescein isothiocyanate-labeled DNA. Binding was initially examined using the forked duplex, which includes single and double strands. In the experiment, the purified WRN winged-helix domain was used as a positive control, which comprises residues 949–1079 (13) known to strongly bind the forked duplex (29). The results are shown in Fig. 6A. No specific interaction of WRN HRDC with the forked duplex was detected even at high protein concentration (50 μM), whereas the winged-helix exhibited strong binding in the nanomolar range (dissociation constant ~100 nM).

The assay was then performed using single-stranded DNA (16-mer), but no interaction was observed with HRDC, whereas the winged-helix exhibited binding with a similar dissociation constant (Fig. 6B). Moreover, we carried out the assays using various DNA structures that represent DNA intermediates generated in the cell, including a blunt-ended duplex, a 5′- or 3′-overhang duplex, a nicked or gapped duplex, a bubble, and a Holliday junction (listed in supplemental Fig. S4). None of these substrates exhibited any detectable interaction with WRN HRDC (data not shown).

The result is consistent with the gel mobility shift assay by von Kobbe et al. (29) using a GST-tagged WRN HRDC fragment, which comprised residues 1072–1236 (the C terminus includes the last HRDC residue, Ser1235), and no detectable binding to the forked duplex was observed. They also reported on the inability of a larger WRN fragment (residues 1072–1432) to bind single-stranded DNA or a blunt-ended duplex (29). In contrast to the strong DNA binding activity of the WRN winged-helix domain, the binding ability of the WRN HRDC domain should be extremely low or at least undetectable in vitro.

**DISCUSSION**

**Inability of WRN HRDC to Bind DNA**—The HRDC domains have been found in proteins involved in DNA metabolism, such as PcrA helicase (33), Rep helicase (34), and DNA polymerase β (35), and in RNA metabolism, such as RNase D (36). Accordingly, interest in the RecQ family HRDC domains has focused on their DNA binding ability (18). In fact, HRDCs from *S. cerevisiae* Sgs1 and *E. coli* RecQ have been demonstrated to interact with DNA in vitro, although their affinity is rather broad and weak (dissociation constant in the micromolar range) (19, 20). The domains are thought to function as auxiliary components to help target the Sgs1 and *E. coli* proteins to DNA substrates in the nucleus (37).

On the other hand, there are no data to support the physical and functional interaction of WRN HRDC with DNA either in vivo or in vitro. Human WRN protein variants that lack the HRDC domain still bind DNA (29). The present study has provided the first report detailing the unique structural features of the WRN HRDC domain, which...
includes the additional terminal motifs stacked together on the domain comprising N-terminal elongated helix-α1 and the C-terminal extended loop. Although WRN HRDC possesses several clusters of basic residues at the bottom of the domain (Fig. 5A, front and back views), neither of the proposed DNA binding sites for Sgs1 or E. coli RecQ are conserved. Consistent with this, the fluorescence polarization assays demonstrated the inability of WRN HRDC to bind DNA.

**Structural Independence of WRN HRDC Domain—**E. coli RecQ can be separated into two fragments following proteolysis, a large catalytic fragment that includes the helicase and winged-helix domains, and a C-terminal fragment comprising the HRDC domain (15). This suggests a central role of the winged-helix domain in catalytic helicase function, and an alternate role for HRDC in the RecQ family. In fact, the HRDC domain is not essential for helicase activity in the RecQ family of proteins and is largely dispensable (reviewed in Ref. 38). In *E. coli*, the HRDC domain is tethered to the winged-helix domain by a short linker composed of 14 residues (20). The linker length in Sgs1 and BLM is difficult to estimate due to the lack of homology and absence of determined structures. The folding of the winged-helix domain has only been verified in *E. coli* (16) and WRN (13). The corresponding linker regions in the other protein families, including PcrA helicase (33), Rep helicase (34), DNA polymerase β (35), and RNase D (36), are also commonly short (<10 residues), as indicated by the crystal structures.

In contrast, WRN is unique in possessing a considerably long linker region comprising 76 residues (1069–1144) that connects HRDC to the winged-helix domain. This region was predicted to be unstructured using structure prediction servers (data not shown). The present study revealed that the WRN HRDC domain separated from the other portions of the protein remains soluble as a monomer and is markedly stable and resistant to high temperature, low and high pH, and protease digestion. This robust feature of WRN HRDC may suggest an independent role of the domain in WRN.

The crystal structure of RNA polymerase III subcomplex C17/25 was recently determined and showed that the complex possesses an HRDC domain at the C terminus of subunit C17, connected by a disordered loop comprising ~25 residues (39). The C17 HRDC domain showed no detectable nucleic acid binding even at high protein concentration and seems to be largely mobile in the subcomplex structure (39). The domain is suggested to be involved in protein-protein interactions with the other subunit of the polymerase. Because the result is partly comparable with that observed for WRN, a question arises as to whether an intrinsic domain-domain interaction exists within WRN so that HRDC can be attracted to the catalytic core. We performed a pull-down assay using purified GST-tagged HRDC and non-tagged winged-helix domains (residues 949–1079), however no interaction between the domains was detected even at the high protein concentration of 50 μM. Although further binding assays using helicase and exonuclease domains are the subject of future investigations, coupled with the robust feature of WRN HRDC domain and its inability to bind DNA, it is speculated that WRN may have evolved the long linker region to separate the HRDC domain from the catalytic core. One advantage with this spatial isolation may have to do with intermolecular protein-protein interactions.

**Possible Binding Partners of WRN HRDC—**The genomes of multicellular eukaryotes encode multiple RecQ homologues, each with specialized functions. Mutation in one recQ gene is not compensated for by the presence of the other homologous proteins, as evidenced by the three human syndromes that arise from individual mutation of three of the five recQ genes. One way in which the RecQ family of proteins is specialized is through their association with distinct sets of cellular proteins (5). Indeed, a number of proteins have been identified that specifically interact with WRN (and/or BLM) in *vitro* as well as in *vivo* (4–6, 9, 11).

In particular, WRN interacts with p53, a tumor suppressor protein that is frequently inactivated in human cancer (40, 41). Werner syndrome cells, in which the C terminus of WRN is truncated, show strong reduction in p53-dependent apoptosis and increased cancer phenotype (42). The WRN-binding site in p53 has been mapped to a 75-residue C-terminal region comprising residues 318–393 (43), which includes the tetramerization domain and negative regulatory domain (reviewed in Refs. 5, 9, 44). Interestingly, the binding region in WRN has been mapped to C-terminal residues 1014–1432 (43, 45). Within these residues, only HRDC is identified as a structurally folded domain, implying that the HRDC domain is responsible for p53 recognition.

Overexpression of *S. cerevisiae* Sgs1 can correct the attenuated p53-mediated apoptotic phenotype in Bloom syndrome cells but not in Werner syndrome cells (42), suggesting that WRN plays a distinct role in human cells that cannot be compensated for by Sgs1 or BLM. In the present study, it was unexpectedly revealed that WRN HRDC possesses a large acidic/hydrophobic patch at the head of the domain (Fig. 5A, front view), which results from binding of the C-terminal extended loop. The motif is specific to WRN and is lacking in both Sgs1 and *E. coli* RecQ. Because the C-terminal region of p53 is rich in basic residues (isoelectric point of 9.7 for residues 318–393), it is tempting to speculate that the acidic/hydrophobic patch on WRN HRDC may play an important role in the interaction with p53.

On the other hand, it was recently demonstrated that WRN quickly accumulates at the site of DNA double-strand breaks (DSBs) both in human (46) and *Xenopus* (47) cells. The DSB contributes significantly to the rate of cancer in humans (48). WRN may play a central role in non-homologous end-joining processes (49, 50). Interestingly, the recruitment event only requires the HRDC domain in WRN (46). The present study demonstrated that WRN HRDC lacks detectable DNA binding ability irrespective of the target DNA structures (Fig. 6 and supplemental Fig. S4), thus it is unlikely that the WRN HRDC directly recognizes the DSB sites. The HRDC domain may interact with one or more other proteins that recognize DSB, thus facilitating concomitant recruitment of WRN to the DSB site. Identification of such a binding partner protein would provide for interesting future investigations.

Moreover, there are two proteins identified as a potential binding partner of WRN HRDC. These are the homologous recombination mediator RAD52 (51) and the p50 subunit of...
DNA polymerase 6 (52), which interact with WRN fragments comprising residues 982–1432 (includes only HRDC as a folded domain) and 949–1401 (includes HRDC and winged-helix), respectively (reviewed in Ref. 11). As a result of two terminal extensions stacked together on the conventional domain, WRN HRDC possesses a significantly larger solvent-accessible surface area (5835 Å²) compared with those of Sgs1 (5229 Å²) and E. coli RecQ (5008 Å²). The increase in surface area would provide WRN with greater potential sites for molecular interactions, such as the hydrophobic pocket found at the back of the domain (Fig. 5A, back view). The pocket region, in which a large residual density (plausibly of a small molecule) has been observed, would be active in hydrophobic interactions and thus may be utilized for binding to a hydrophobic residue from the other partner proteins.

Conclusion—In this report, we presented the first structure of the human WRN HRDC domain. Compared with HRDC structures of unicellular organisms, including S. cerevisiae Sgs1 and E. coli RecQ, WRN HRDC possesses additional terminal residues stacked on top of the domain. The surface property of WRN HRDC is distinct, in which neither of the DNA binding sites for Sgs1 or E. coli is conserved, and WRN HRDC was shown to lack DNA binding ability in vitro. The domain may be adapted to play a different role from that of the unicellular RecQ helicases, such as binding to other proteins, including p53.

Among the five mammalian RecQ proteins, only WRN and BLM share the presence of an HRDC domain. In BLM, the domain is a critical determinant for the efficient binding to and unwinding of double Holliday junctions with topoisomerase IIIα (53, 54). The activity is specific to BLM, and WRN cannot substitute for BLM in the dissolution reaction. Heterogeneity in HRDC domains (WRN and BLM HRDCs shares only 19% identity) may correlate with functional differences in WRN and BLM. Most of the mutations identified so far in Werner patients result in a truncated WRN protein that lacks the C-terminal residues, including HRDC and nuclear localization signal (55). In addition to the critical loss of nuclear localization signal, disruption of HRDC-mediated molecular interactions may also be related with the onset of disease. Further biochemical and cell biological experiments are required to determine the precise roles played by the WRN HRDC domain in vivo.

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