Identification and Biochemical Characterization of a Werner’s Syndrome Protein Complex with Ku70/80 and Poly(ADP-ribose) Polymerase-1*

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Werner’s syndrome (WS) is an inherited disease characterized by genomic instability and premature aging. The WS gene encodes a protein (WRN) with helicase and exonuclease activities. We have previously reported that WRN interacts with Ku70/80 and this interaction strongly stimulates WRN exonuclease activity. To gain further insight on the function of WRN and its relationship with the Ku heterodimer, we established a cell line expressing tagged WRN16, a WRN point mutant lacking helicase activity, and used affinity purification, immunoblot analysis and mass spectroscopy to identify WRN-associated proteins. To this end, we identified three proteins that are stably associated with WRN in nuclear extracts. Two of these proteins, Ku70 and Ku80, were identified by immunoblot analysis. The third polypeptide, which was identified by mass spectrometry analysis, is identical to poly(ADP-ribosyl) polymerase-1 (PARP-1), a 113-kDa enzyme that functions as a sensor of DNA damage. Biochemical fractionation studies and immunoprecipitation assays and studies confirmed that endogenous WRN is associated with subpopulations of PARP-1 and Ku70/80 in the cell. Protein interaction assays with purified proteins further indicated that PARP-1 binds directly to WRN and assembles in a complex with WRN and Ku70/80. In the presence of DNA and NAD+, PARP-1 poly(ADP-ribosyl)ates itself and Ku70/80 but not WRN, and gel-shift assays showed that poly(ADP-ribosylation) of Ku70/80 decreases the DNA-binding affinity of this factor. Significantly, (ADP-ribosylation) of Ku70/80 reduces the ability of this factor to stimulate WRN exonuclease, suggesting that covalent modification of Ku70/80 by PARP-1 may play a role in the regulation of the exonuclease activity of WRN.

Werner’s syndrome (WS)† is a human genetic disease with many features of premature aging (1, 2). The first signs of this disorder appear soon after puberty, with the symptoms becoming fully evident in individuals between 20 and 30 years old. Individuals with WS display a high incidence of diseases associated with normal aging, including atherosclerosis, osteoporosis, type II diabetes mellitus, and cancer. Myocardial infarction and cancer are the most common causes of death among WS patients. The median age of death is ~47 years (1, 3). Cells isolated from WS patients show genomic instability and a shorter replicative life span (4). The genomic instability is characterized by an elevated rate of chromosomal translocations and extensive genomic deletions (5). These findings suggest that genomic instability underlies the development of the diseases associated with WS. Cultured cells from WS patients are also hypersensitive to some DNA damaging agents (4), suggestive of a defect in the repair of specific DNA lesions.

Werner’s syndrome is caused by mutations within a single gene, which is located on chromosome 8 (6). The cDNA encodes a protein (Werner’s syndrome protein, WRN) with strong homology to a class of enzymes known as RecQ helicases (7). In addition, the amino-terminal region of WRN is highly homologous to the nuclease domain of Escherichia coli DNA polymerase I and ribonuclease D (8). Helicase and exonuclease activities with a 3′ to 5′ directionality have been demonstrated in vitro using recombinant WRN (9–14). A nuclear localization signal is found near the carboxyl-terminal end of WRN (4). All of the WRN mutations in individuals with Werner’s syndrome result in non-sense mutations or frame-shifts leading to truncated proteins. The prevailing hypothesis is that the aberrant proteins do not enter the nucleus and are rapidly degraded. Consistent with this idea, cell lines from WS patients show no detectable WRN polypeptide (15).

A number of studies have indicated that WRN binds to proteins that are involved in DNA replication and repair, such as the replication protein A, topoisomerase I, DNA polymerase δFen-1, p53, proliferating cell nuclear antigen, and Rad52 (11, 16–22). Although some of these proteins have been shown to influence WRN catalytic activities in vitro, the physiological significance of these interactions remains largely unknown.

In previous studies, we reported that WRN binds to Ku70/80 heterodimer (Ku) (23), a factor involved in the repair of double-strand DNA breaks by non-homologous end joining (reviewed in Ref. 24). Remarkably, our studies showed that Ku recruits WRN to DNA ends and alters the properties of the WRN exonuclease (23, 25). Other studies have also indicated that Ku70/80 is required for the WRN-mediated hydrolysis of DNA molecules containing lesions mimicking oxidative DNA damage (26). A functional interaction between WRN and Ku70/80 is also supported by genetic studies showing that Ku80-null mice display genomic instability and shortened life span (27–31). Thus, biochemical and genetic evidence suggest that Ku80 and WRN may function together in a DNA repair pathway required for the maintenance of genome integrity.

In this study, we report the identification of a cellular WRN complex composed of WRN, Ku70/80, and poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a nuclear factor implicated in

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‡ The abbreviations used are: WS, Werner’s syndrome; WRN, Werner’s syndrome protein; Ku, Ku70/80 heterodimer; PARP-1, poly(ADP-ribose) polymerase-1, IRES-GFP, internal ribosome entry site-green fluorescent protein.

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the control of genomic stability and mammalian life span (32, 33). Our results indicate that a subpopulation of PARP-1 co-elutes over ion-exchange and gel-filtration chromatography and coimmunoprecipitates with WRN and Ku70/80. Further biochemical analyses show that PARP-1 poly(ADP-ribosylates Ku70/80 but not WRN in vitro, and ADP-ribosylation of Ku70/80 reduces its DNA-binding activity and weakens its ability to stimulate the exonucleolytic activity of WRN.

**EXPERIMENTAL PROCEDURES**

**Lentiviral Vectors and Stable Cell Lines—**We subcloned Flag-tagged wild-type and helicase mutant (WRN)N, K577M, A → O transition at position 17390 of WRN open reading frame (Ref. 34) into WRN cDNAs (excluding the polyadenylation signal and 3′ untranslated sequences) into the polylinker sequence of a lentiviral transfer vector pRsLsin.hCMV-Puro. To generate pRsLsin.hCMV-Puro, the internal ribosome entry site-green fluorescent protein (IREs-GFP) region of the vector pRsLsin.hCMV-IREs-GFP (35) was replaced by a minimal SV40 promoter driving the expression of the pyruvycin N-acetyl transaminase gene. We produced recombinant lentiviruses by transfection of three-plasmid transient co-transfection of human 293T cells as described by Naldini et al. (36). For lentiviral infection, 293T cell cultures were trypsinized, seeded onto 60-mm plates, and incubated at 37 °C for 24 h. The supernatant containing viral particles was collected and added to the 293T cell cultures that were 30–40% confluent. After a 6-h incubation at 37 °C, the supernatant was removed, the cells were washed twice and incubated in Dulbecco’s modified Essential medium containing 10% serum at 37 °C. Transduced cells expressing Flag-WRN and Flag-WRNN were selected in media supplemented with puromycin (10 μg/ml). The expression of Flag epitope-tagged proteins was analyzed by immunoblotting with anti-Flag antibodies (Sigma).

**Construction of Plasmids and Production of Recombinant Proteins—**Recombinant WRN and Ku70/80 heterodimer were purified from baculovirus-infected Sf9 cells as described previously (23). The pVL1392 Flag-PARP-1 vector for expression of the recombinant Flag-PARP-1 protein in Sf9 cells was constructed by PCR cloning. First, the region of the open reading frame from amino acids 1–232 was amplified from a PARP-1 cDNA clone (Open Biosystems) by PCR using the following primers: 5′-CTAGGGGCTATAGGCGGAGTCTTC-3′/GGTCTTTTCC-AAGCTTACTACCTC-3′. The amplified fragment was digested with NdeI and HindIII and subcloned into the pCDNA3/HisA.Flag-PARP-1-N. The full-length PARP-1 cDNA was then subcloned into the NdeI-HindIII sites of a pVL1392-Flag vector. pVL1392-Flag-PARP-1 was cotransfected with linearized baculovirus BaculGold, Pharamingen) into Sf9 cells to generate the recombinant baculovirus. For the purification of recombinant Flag-PARP-1, baculovirus-infected cells were lysed in lysis buffer (10 μM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet P-40). Flag-PARP-1 was purified by chromatography on DEAE-Sepharose and anti-Flag resin columns.

**Purification of the WRN Complex—**100 μg of nuclear extracts prepared from cells expressing Flag-WRNN were incubated with anti-Flag beads at 4 °C for one hour. After extensive washes, bound proteins were eluted with BCO buffer (1 M KCl, 10 mM Tris HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol, and 1.0 mM ADP-ribosylation of PARP-1, and Ku70/80) immunoblots; 100 μl for PARP-1 and Ku70/80 immunoblots; 100 μl for WRN immunoblot) from alternate fractions were analyzed by SDS-PAGE and immunoblotted with antibodies raised against WRN and Ku70. For the analysis of WRN, 100 μl of the indicated fractions were subjected to trichloroacetic acid precipitation prior to loading on the SDS-polyacrylamide gel. Likewise, the 0.4 M KCl eluate from the SP-Sepharose column (SP-0.4) was loaded onto an 8–16% polyacrylamide gel and analyzed by immunoblotting with WRN, Ku70, and PARP-1 antibodies. The flow-through from the SP-Sepharose column (SP-0.15 Superose, 90 ml). The column was run as described above. Aliquots (15 μl for PARP-1 and Ku70/80 immunoblot; 100 μl for WRN immunoblot) from alternate fractions were analyzed by SDS-PAGE and immunoblotted with antibodies raised against WRN and Ku70. For the analysis of WRN, 100 μl of the indicated fractions were subjected to trichloroacetic acid precipitation prior to loading on the SDS-polyacrylamide gel. Likewise, the 0.4 M KCl eluate from the SP-Sepharose column (SP-0.4) was dialyzed in buffer A containing 0.15 M KCl, centrifuged at 100,000 × g for 60 min, and 1 ml of the supernatant was loaded onto a Superose 6 column (SP-0.15 Superose, 90 ml). The column was run as described above. Aliquots (15 μl for PARP-1 and Ku70/80 immunoblot; 100 μl for WRN immunoblot) from alternate fractions were analyzed by SDS-PAGE and immunoblotted with antibodies raised against WRN and Ku70.
resolved by 16% polyacrylamide-urea gel electrophoresis, visualized by autoradiography and phosphoimager analyzer, and quantified with ImageQuant software (Molecular Dynamics).

Electrophoretic Mobility Shift Assay—A 20-mer A1 was labeled using [γ-32P]ATP and T4 polynucleotide kinase and then annealed to a partially complementary 46-mer A2. Radiolabeled double-strand oligonucleotides (80 fmol, 200,000 cpm) were incubated with increasing amounts (100–300 fmol) of unmodified Ku70/80 or poly(ADP-ribosyl)ated Ku70/80 in 15 μl of buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, and 10% glycerol) at 25 °C for 10 min. The samples were resolved by electrophoresis at 10 V/cm through a 4% polyacrylamide gel at 4 °C. The gel was dried, and reaction products were visualized by autoradiography and Phosphoimager analyzer and quantified with ImageQuant software (Molecular Dynamics).

(ADP-ribosylation of Proteins—20 μg of purified Flag-PARP-1 was incubated with 2 μg His-Ku70/80 or His-WRN in a reaction mixture (500 μl) containing 100 mM Tris pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, 20 ng/μl sonicated salmon sperm DNA, and 20 μM NAD+. After incubation at 30 °C for 2 h, the salts concentration in the reaction was adjusted to 400 mM KCl, and the reaction mixture was incubated with anti-Flag beads at 4 °C for 1 h. The supernatant was collected and incubated with metal affinity resin (Talon, Clontech) at 4 °C for one hour. After extensive washes with 20 mM Tris, pH 8.0, 150 mM KCl, 1 mM MgCl2, 10% glycerol, and a mixture of protease inhibitors, Ku70/80 or WRN were eluted with buffer containing 100 mM imidazole, dialyzed, and analyzed by SDS-polyacrylamide gel electrophoresis, silver staining, and immunoblotting, or snap frozen at −80 °C.

RESULTS

Isolation of WRN-associated Proteins from Cells Stably Expressing Flag-WRN$^\text{H}$—To assist in the purification of a native WRN protein complex, we generated stable 293T cells using recombinant, replication-defective, lentivirus vectors expressing either wild-type Flag-WRN or Flag-WRN$^\text{H}$, a WRN protein carrying a point mutation (K577M) that inactivates the helicase activity (25, 34). Because Flag-WRN is expressed at extremely low levels (Fig. 1A, lane 1), this cell line is not particularly useful for biochemical studies. One possible explanation for this effect is that the increased level of functional WRN may be toxic to the cell. On the other hand, the level of expression of Flag-WRN$^\text{H}$ (lane 2), which is comparable with that of the endogenous WRN (data not shown), is sufficient for the isolation and biochemical characterization of WRN$^\text{H}$ from cell extracts. Thus, we prepared nuclear extracts from the 293T-WRN$^\text{H}$ cells and purified the tagged protein by affinity chromatography on anti-Flag resin. In parallel, extracts from 293T cells that were infected with a control lentivirus were subjected to the same purification procedure. Proteins bound to the affinity column were eluted with high salts and examined by SDS-polyacrylamide gel electrophoresis and silver staining. This analysis revealed the presence of three polypeptides of 70, 90, and 120 kDa, respectively. These proteins were eluted

![Figure 1](image1.png)

**Fig. 1.** Identification of a WRN protein complex in cells expressing Flag-WRN$^\text{H}$. A, lysates were prepared from 293T cells transduced with lentiviruses expressing Flag-WRN (lane 1), Flag-WRN$^\text{H}$ (lane 2), or no protein (lane 3), analyzed by SDS-PAGE, and immunoblotted with antibody against the Flag tag. Lane 4, purified recombinant Flag-WRN. B, cell extracts prepared from a 293T cell line stably expressing Flag-WRN$^\text{H}$, a helicase mutant WRN (lanes 2 and 4), and the parental 293T cells (lanes 1 and 3) were incubated with anti-Flag beads; after extensive washes, the bound proteins were eluted from the beads with BCO buffer (see “Experimental Procedures”). Under these conditions, the antigen (Flag-WRN$^\text{H}$) and the non-specific binding proteins remain bound to the beads, whereas the proteins associated with Flag-WRN$^\text{H}$ are eluted. Eluted proteins (lanes 1 and 2) and proteins that remained bound to the beads (lanes 3 and 4) were separated by SDS-8% PAGE and analyzed by silver staining. Arrow, novel WRN-associated factor. C, eluates from the control (lane 1) and Flag-WRN (lane 2) immunopurification reactions were resolved by SDS-8% PAGE and analyzed by immunoblotting with anti-PARP-1 (top panel) and anti-Ku70 and anti-Ku80 (bottom panel) antibody.

![Figure 2](image2.png)

**Fig. 2.** WRN binds to PARP-1 in vivo and in vitro. A, PARP-1 and Ku70/80 are coimmunoprecipitated with WRN from cell extracts. Nuclear extracts from 293T cells were subjected to immunoprecipitation with anti-WRN (lane 3) or anti-β-actin (lane 2) antibodies. Immunoprecipitation products were analyzed by Western blotting with anti-WRN (top panel), anti-PARP-1 (middle panel), and a mixture of anti-Ku70 and anti-Ku80 (bottom panel) antibodies. Lane 1 contains purified recombinant WRN, PARP-1, and Ku70/80 as controls. Lane 4 contains 10% of the extract used in the immunoprecipitation. B, in vitro protein-protein interactions between WRN, PARP-1, and Ku70/80. Purified Flag-PARP-1 (2.0 μg) (lanes 6 and 8) or Flag-HCV polymerase (2.0 μg) (negative control: lanes 5 and 7) were immobilized on anti-Flag beads and then incubated with 300 μ (2.0 μg) of purified His-WRN alone (lane 6) or a mixture containing purified His-WRN and Ku70/80 (lane 8). After several washes, the bound proteins were solubilized in SDS-containing buffer and analyzed by immunoblotting with anti-WRN (top panel), anti-Flag (middle panel), and a mixture of anti-Ku70 and anti-Ku80 antibodies. Lanes 1–4 show 5% of the protein inputs used in the protein-binding assay.
FIG. 3. Fractionation of WRN, PARP-1, and Ku70/80 by ion-exchange and gel-filtration column chromatography. A, graphic representation of the fractionation scheme used to analyze WRN, PARP-1, and Ku70/80 complex formation. B, nuclear extracts from 293 cells were fractionated on DEAE-Sepharose and SP-Sepharose as described under “Experimental Procedures.” The nuclear extract (15 μl, lane 1), DEAE flow-through (15 μl, lane 2), SP-Sepharose 0.15 M KCl eluate flow-through (15 μl, lane 3) and 0.4 M KCl eluate (15 μl, lane 4) were analyzed by immunoblotting with anti-WRN, anti-PARP, and anti-Ku70 antibodies. C, the 0.15 M KCl eluate (containing WRN and Ku70/80) and the 0.4 M KCl eluate (containing WRN, PARP-1, and Ku70/80) from the SP-Sepharose column were independently fractionated on Superose 6 columns (SP-0.15 superose 6, top panel; SP-0.4 superose 6, bottom panel) as described under “Experimental Procedures.” Indicated fractions (fr.#) were analyzed by SDS-PAGE and immunoblotted with antibodies against WRN, PARP, and Ku70. Peak fractions for each factor were run on the same gel to eliminate possible differences between gels or membranes. The position of the peaks of elution of the molecular mass standards (thyroglobulin and....
To further examine the relationship between WRN and PARP-1, we performed immunoprecipitation analysis. As shown in Fig. 1, WRN binds to PARP-1 in the presence of Ku70/80 (lanes 4–6). PolyADP-ribosylation of PARP-1 was detected by silver staining of the gel. WRN and Ku70/80 were purified from insect cells infected with recombinant baculoviruses expressing His-WRN or His-Ku70 and -Ku80. WRN and Ku70/80 were separately incubated with PARP-1 in the presence of sonicated DNA and NAD⁺ (WRN, lanes 2 and 6; Ku70/80, lanes 4 and 8) or buffer only (WRN, lanes 3 and 7; Ku70/80, lanes 5 and 9). WRN and Ku70/80 were collected from the respective reaction mixtures by incubation with metal affinity resin, solubilized in SDS-containing buffer, resolved by SDS-PAGE, and analyzed by silver staining (lanes 2–5) and immunoblotting with anti-poly(ADP-ribose) antibody (lanes 6–9). Lane 1 contains a molecular mass marker standard (HMW standard, Bio-Rad).

**Physical Interaction between WRN and PARP-1 in Human Cells**—To provide further evidence that PARP-1 interacts with endogenous WRN in vitro, we immunoprecipitated WRN from nuclear extracts prepared from 293T cells using anti-WRN antibodies. As a control, the same nuclear extract was subjected to immunoprecipitation with antibody against β-actin. The products of the immunoprecipitation reactions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. The results indicate that PARP-1 communoprecipitates with WRN but not with β-actin (Fig. 2A). The Ku70/80 complex is also detected in the immunoprecipitation reaction with anti-WRN antibodies, confirming the results of the immunopurification of Flag-WRN. Treatment of the nuclear extract with DNase I prior to the immunoprecipitation yielded identical results (data not shown).

Previous studies have shown that PARP-1 binds to Ku70/80 (38, 39); therefore, it is possible that Ku70/80 mediates the interaction between WRN and PARP-1. To determine whether WRN binds directly to PARP-1, recombinant Flag-PARP-1 was immobilized on anti-Flag beads and incubated with recombinant WRN (His-WRN). In parallel reactions, immobilized Flag-PARP1 was incubated with Ku70/80 or with a mixture containing Ku70/80 and WRN. After washing the beads, the bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with antibodies against WRN, Ku70/80, and PARP-1. As shown in Fig. 2B, WRN binds to PARP-1 in the absence of Ku70/80 (lane 6), suggesting that the interaction between WRN and PARP-1 in the WRN complex is mediated, at least in part, by direct interaction between these two proteins. Similarly, WRN binds to PARP-1 in the presence of Ku70/80 (lane 8). These results suggest that these factors can individually associate with each other and can form a trimeric complex in vitro.

**Analysis of the WRN Complex by Ion Exchange and Gel Filtration Chromatography**—To further examine the relationship among WRN, Ku70/80, and PARP-1 and determine whether there is evidence for the existence of a stably associated complex in vivo, we performed a three-step fractionation of the nuclear extracts using a DEAE ion-exchange column, SP-Sepharose ion-exchange column, and Superose 6 gel-filtration columns (Fig. 3). The flow-through of the DEAE column, which contains PARP-1, WRN, and Ku70/80 as detected by immunoblotting (Fig. 3B, lane 2), was applied to an SP-Sepharose column. Immunoblot analysis of the fractions from the step-eluted SP-Sepharose column indicates that WRN and Ku70 elute in both the flow-through (SP-0.15) (lane 3) and the 0.4 M KCl fraction (SP-0.4) (lane 4), whereas PARP-1 elutes in the 0.4 M KCl fraction (SP-0.4) (lane 4). The SP-Sepharose flow-through (SP-0.15) and 0.4 M KCl fraction (SP-0.4) were then individually fractionated further on Superose 6 gel-filtration columns. The analysis of the fraction eluted from the Superose 6 column loaded with the SP-Sepharose flow-through fraction (SP-0.15 Superose 6) indicates that WRN and a portion of Ku coelute in fractions 44–50 (Fig. 3C, top panel). The immunoblot analyses of fractions 12–20 of the SP-0.4 Superose 6 column and fractions 64–70 of the SP-0.15 Superose 6 column did not reveal any detectable amounts of WRN, Ku70, and PARP-1 and are not shown. Bracketed fractions represent the fractions used in the immunoprecipitation reactions shown in panel B. D, communoprecipitation of WRN, PARP-1, and Ku70/80 from gel-filtration column fractions. We combined the SP-0.15 Superose 6 column fractions 45/46 and fractions 47/48 (lanes 1 and 2, respectively) and the SP-0.4 Superose 6 column fractions 45/46 and fractions 47/48 (lanes 3 and 4, respectively). Pooled fractions were individually incubated with anti-WRN antibody and subjected to immunoprecipitation with protein A-Sepharose. Immunoprecipitated proteins were analyzed by immunoblotting with anti-WRN (top panel), anti-PARP-1 (middle panel), and anti-Ku70 (bottom panel) antibodies.
Poly(ADP-ribosyl)ation of Ku70/80 impairs DNA binding and reduces the Ku-mediated stimulation of WRN exonuclease activity. A. Ku70/80 was purified from insect cells coinfected with recombinant baculoviruses expressing human His-Ku70 and -Ku80. Ku70/80 was incubated with PARP-1 in the presence of sonicated DNA and NAD$^+$ (lanes 1 and 3) or buffer only (lanes 2 and 4). Ku70/80 were collected by incubation with a metal ion resin, solubilized in SDS-containing buffer, resolved by SDS-PAGE, and analyzed by silver staining (lanes 1 and 2) and immunoblot (α-PAR) (lanes 3 and 4).
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A similar analysis of the fractions eluted from the Superose 6 column loaded with the SP-Sepharose 0.4 M KCl fraction (SP-0.4 Superose 6) shows that WRN coelutes with a portion of PARP-1 and Ku in fractions 44–52 (Fig. 3C, bottom panel). Note that these data are only suggestive of complex formation. Complex formation per se was then tested by direct immunoprecipitation of these fractions by using anti-WRN antibodies and by probing the precipitated proteins for immunoreactivity against WRN, PARP-1, and Ku70. As shown in Fig. 3D, Ku70 coimmunoprecipitated with WRN from pooled fractions 45/46 and fractions 47/48 of the SP-0.15 Superose 6 column (Fig. 3D, lanes 1 and 2), and Ku70 and PARP-1 coimmunoprecipitated with WRN from pooled fractions 45/46 and fractions 47/48 of the SP-0.4 Superose 6 column (lanes 3 and 4). These results suggest that two WRN complexes may exist in vivo, one with Ku and another with both Ku and PARP-1.

Poly(ADP-ribosyl)ation Reduces Ku70/80 DNA-binding Activity—PARP-1 is a nuclear protein that binds to DNA strand breaks and catalyzes ADP-ribosylation of itself and other nuclear proteins by using NAD⁺ as a cofactor. This catalytic activity requires DNA. To establish that our preparation of purified recombinant PARP-1 was able to poly(ADP-ribosyl)ate itself in a DNA-dependent manner, we incubated recombinant PARP-1 in the absence or presence of increasing amounts of DNA and NAD⁺. As shown in Fig. 4A, recombinant PARP-1 becomes poly(ADP-ribosyl)ated in the presence but not in the absence of DNA, as indicated by the appearance of high molecular mass products in the silver-stained gel (compare Fig. 4A, lanes 1 and 2 with lane 3). Importantly, poly(ADP-ribosyl)ation of PARP-1 depends on the presence of NAD⁺, as incremental formation of high molecular mass products is observed in the presence of increasing amounts of NAD⁺, and the omission of NAD⁺ from the reaction mixture prevents the formation of these products (Fig. 4A, lanes 4–7). To determine whether WRN and Ku70/80 were substrates of the poly(ADP-ribose)-polymerase activity of PARP-1, purified WRN and Ku70/80 were individually incubated with PARP-1 in the presence of NAD⁺ and DNA. WRN and Ku70/80 were then immunoprecipitated from the respective reaction mixtures, analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with a monoclonal antibody that recognizes poly(ADP-ribosyl)ation (anti-PAR antibody). The results of this experiment indicate that PARP-1 poly(ADP-ribosyl)ates Ku70/80 (Fig. 4B, lane 8) but not WRN (Fig. 4B, lane 6). Interestingly, the apparent molecular mass of Ku70 and Ku80 does not change significantly after covalent modification, suggesting that only a few ADP-ribose molecules are added to both Ku70 and Ku80.

Poly(ADP-ribosyl)ation Inhibits Ku70/80 DNA-binding Activity and Reduces the Ku-dependent Stimulation of WRN Exonuclease Activity—The addition of ADP-ribose molecules increases the negative charge of a protein, which is thought to alter the DNA-binding properties of the target factor. To determine whether ADP-ribosylation influences the DNA-binding activity of Ku70/80, we purified in vitro poly(ADP-ribosyl)ated Ku70/80 and unmodified Ku70/80. Ku70/80 was incubated with PARP-1 in the presence of DNA and NAD⁺ to allow poly(ADP-ribosyl)ation and purified by affinity chromatography. The purity of unmodified and poly(ADP-ribosyl)ated Ku70/80 was determined by silver staining of SDS-polyacrylamide gels (Fig. 5A, lanes 1 and 2), and poly(ADP-ribosyl)ation was confirmed by immunoblot analysis with anti-PAR antibody (lanes 3 and 4). Increasing amounts of unmodified or poly(ADP-ribosyl)ated Ku70/80 (Fig. 5A) were incubated with a radiolabeled double-strand oligomer, and DNA-binding activity was analyzed by electrophoretic mobility shift assays (Fig. 5, A and C). The results of this experiment indicate that ADP-ribosylation significantly reduced the DNA-binding activity of Ku70/80 (compare lanes 2–4 to 5–7), suggesting that this covalent modification alters the DNA-binding properties of this factor.

We have shown previously that Ku70/80 recruits WRN to DNA ends and stimulates WRN exonuclease activity. Because PARP-1 has been reported to bind to DNA ends (40), we examined whether PARP-1 may influence WRN exonuclease activity. We performed WRN exonuclease assays with PARP-1 in the presence and absence of Ku70/80 and found that unmodified PARP-1 or poly(ADP-ribosyl)ated PARP-1 do not affect the exonuclease activity of WRN nor alter the strong stimulation of this activity by Ku70/80 (data not shown). Then we wanted to determine whether poly(ADP-ribosyl)ation of Ku70/80 influences WRN exonuclease activity. Because the experimental conditions used for the exonuclease assay are not suitable for PARP-1 poly(ADP-ribosyl)ation activity, Ku70/80 was poly(ADP-ribosyl)ated in vitro prior to its addition to the exonuclease reaction (Fig. 5A). WRN was then incubated with a radiolabeled double-strand oligonucleotide in exonuclease buffer in the presence of poly(ADP-ribosyl)ated Ku70/80 or unmodified Ku70/80. The products of these reactions were separated by denaturing acrylamide gel electrophoresis and analyzed by autoradiography and PhosphorImager analyzer. The results of these experiments show that poly(ADP-ribosyl)ated Ku70/80 leads to a modest increase in DNA hydrolysis as compared with the unmodified factor (Fig. 5, panels D and E), indicating that poly(ADP-ribosyl)ation reduces the ability of Ku70/80 to stimulate the exonuclease activity of WRN.

DISCUSSION

In this study, we purified a WRN complex by affinity chromatography and identified its components by immunoblot analysis and mass spectroscopy. We found that WRN resides in a complex with Ku70/80 and PARP-1. Coimmunoprecipitation assays indicated that PARP-1 binds to WRN and Ku70/80 in vivo, and in vitro protein-binding studies with purified factors show that the interaction between PARP-1 and WRN is direct and that WRN can form a trimeric complex with PARP-1 and Ku. Two groups (von Kobbe et al. and Adelfalk et al., Refs. 41 and 42, respectively) have reported an interaction between WRN and PARP-1 while this paper was in review.

immunoblotting with anti-poly(ADP-ribosyl)ated (α-PAR) antibody (lanes 3 and 4). B, poly(ADP-ribosyl)ated Ku70/80 binds to DNA less efficiently than unmodified Ku70/80. 32P-labeled 5′-20-mer(A1/46-merA2) DNA substrate was incubated with 100–300 fmol of Ku70/80 (lanes 3–4) and poly(ADP-ribosyl)ated Ku70/80 (lanes 5–7) at room temperature for 10 min. The reactions were analyzed by 4% native polyacrylamide gel electrophoresis, and the DNA-protein complexes were visualized by autoradiography (lane 1, DNA probe only; lane 2, 100 fmol WRN; lanes 3–5, 100 fmol of WRN and 50, 100, and 200 fmol of Ku70/80, respectively; lanes 6–8, 100 fmol of WRN and 50, 100, and 200 fmol of poly(ADP-ribosyl)ated Ku70/80, respectively). The relative WRN exonuclease activity in the presence of increasing amounts of unmodified or ADP-ribosylated Ku70/80 (panel D) is plotted in a bar graph as a percentage of bound DNA probe. D, poly(ADP-ribosyl)ation of Ku70/80 reduces the stimulation of WRN exonuclease activity. 100 fmol of purified WRN and 50, 100, and 200 fmol of purified Ku70/80 and poly(ADP-ribosyl)ated Ku70/80 were incubated with a 3′-recessed, 32P-labeled 5′-20-mer/46-mer DNA substrate at room temperature for 20 min. Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, DNA probe only; lane 2, 100 fmol WRN; lanes 3–5, 100 fmol of WRN and 50, 100, and 200 fmol of Ku70/80, respectively; lanes 6–8, 100 fmol of WRN and 50, 100, and 200 fmol of poly(ADP-ribosyl)ated Ku70/80, respectively). E, the relative WRN exonuclease activity in the presence of increasing amounts of unmodified or ADP-ribosylated Ku70/80 (panel D) is plotted in a bar graph as 3′ nucleotides removed from the DNA substrate.
fication of Ku70/80 as one of the components of the WRN complex is consistent with previous studies (23, 25, 43), which established Ku70/80 as a functional partner of WRN and suggested a possible role for WRN in DNA repair. The finding that PARP-1, a factor implicated in the cellular response to DNA damage, is a component of the WRN complex supports the idea that WRN may be involved in a pathway that monitors genome integrity. This interpretation is in agreement with a recent genetic study, which showed that double knockout mice lacking WRN and PARP-1 display chromosomal instability and shorter life span (44).

PARP-1 is a highly conserved eukaryotic protein that binds to single- and double-strand DNA breaks and is thought to function as a sensor of DNA damage (32, 33). In response to DNA damage induced by ionizing radiation, alkylating agents, PARP-1 catalytic activity, which utilizes NAD+ to catalyze the addition of multiple ADP-ribose molecules to acceptor proteins. A number of studies have reported that activated PARP-1 poly(ADP-ribose)lates proteins such as histones, p53, topoisomerases, laminas, and PARP-1 itself (45–50). Poly(ADP-ribose)lation of p53 has been reported to influence the DNA-binding activity of this tumor-suppressor protein (51). Moreover, poly(ADP-ribose)lation of chromosomal proteins has been proposed to alter the nucleosomal structure near the DNA strand breaks and to promote the access of repair enzymes to these sites (52). We have examined whether WRN and Ku70/80 are substrates of PARP-1 enzymatic activity and have shown that PARP-1 poly(ADP-ribose)lates Ku70 and Ku80 but not WRN. In addition, our analysis indicates that poly(ADP-ribo- sylation) of Ku70/80 alters the DNA-binding activity of this factor and inhibits the Ku70/80-mediated stimulation of WRN exonuclease activity. Thus, these results indicate that, through covalent modification of Ku70/80, PARP-1 modulates WRN exonuclease activity. Conversely, we did not observe any significant alteration of WRN helicase activity by PARP-1 in the presence or absence of Ku70/80 (data not shown). Given the role of Ku70/80 and PARP-1 in the recognition of DNA breaks, our findings suggest that WRN, once recruited to the site of DNA damage, may participate directly in the processing and resolution of the broken DNA ends. Because there are several-fold more Ku and PARP-1 molecules than WRN in a cell, it is conceivable that WRN in the context of this complex functions as a nucleating factor that organizes a subset of Ku70/80 and PARP-1 molecules into a DNA damage sensory complex involved in the surveillance of genome integrity.

Our fractionation studies indicate that WRN co-elutes with subpopulations of Ku70/80 and PARP-1. It is important to note that coincidence of the peaks of each factor in the gel-filtration chromatography is not expected, as this would suggest that the majority of all three proteins exist in a complex. Such a result is not anticipated, as it is known that all three proteins interact with different partners in response to different physiological cues (24, 53, 54). Importantly, to verify that complex formation was not an artifact of the three factors independently binding to DNA, immunoprecipitation experiments were carried out in the presence of DNase I. These experiments yielded identical results (data not shown). The immunoprecipitation of WRN from eluates of fractionated extracts show that WRN forms two complexes with Ku, one of which contains PARP-1. These findings may indicate that there are two distinct WRN-Ku complexes in the cell. Alternatively, the association of PARP-1 with Ku70/80 and WRN may be part of a dynamic process that involves the rapid assembly and disassembly of this complex in the cell in response to diverse physiological and pathological stimuli. Clearly, we cannot rule out that the two observed WRN-Ku complexes may only be a consequence of the fractionation experiments; future studies will address this issue.

PARP-1 has been implicated in the protection of genome integrity by facilitating the repair of damaged DNA. In addition, PARP-1 participates in the execution of the apoptotic and necrotic pathways in cells with excessive DNA damage (55–57). The necrotic function of PARP-1 has been linked to overactivation of the enzymatic activity, which leads to the cellular depletion of NAD+ and ATP (32, 54, 55). Excessive PARP-1 activity has been implicated in the pathogenesis of several clinical conditions such as stroke, myocardial infarction, arthritis, diabetes, and neurodegenerative disorders (54). The molecular mechanisms that regulate PARP-1 survival and death-promoting effects are poorly defined; thus, it is possible that WRN and Ku70/80 may play a role in the selection of the specific pathway that is activated in response to DNA damage.

PARP-1 and poly(ADP-ribose)ylation have been linked to mammalian longevity, as differences in the catalytic activity of PARP-1 correlate with differences in life span between short and long-lived species (58). Because WS is a premature aging disease, and inactivation of Ku in mice leads to premature aging (27, 28, 31), the identification of a physical interaction between WRN, Ku70/80, and PARP-1 suggests that these proteins are caretakers that function together in a cellular pathway that monitors the integrity of the genome and the longevity potential of an organism. Future biochemical and genetic studies will help in elucidating the link between the WRN complex and the network of cellular pathways that control the fate of cells with damaged DNA and shortened life spans.

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