Ku complex interacts with and stimulates the Werner protein

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Werner syndrome (WS) is the hallmark premature aging disorder in which affected humans appear older than their chronological age. The protein WRNp, defective in WS, has helicase function, DNA-dependent ATPase, and exonuclease activity. Although WRNp functions in nucleic acid metabolism, there is little or no information about the pathways or protein interactions in which it participates. Here we identify Ku70 and Ku86 as proteins that interact with WRNp. Although Ku proteins had no effect on ATPase or helicase activity, they strongly stimulated specific exonuclease activity. These results suggest that WRNp and the Ku complex participate in a common DNA metabolic pathway.

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Werner syndrome [WS] is a human premature aging disorder characterized by the early display of many of the signs and symptoms that are associated with the normal aging process. Cells from WS patients also show a number of phenotypic changes typical of chronologically older normal cells including increased chromosomal abnormalities and rapid onset of cellular senescence (Martin 1997). WS cells are not generally sensitive to DNA-normalities and rapid onset of cellular senescence (Marcus et al. 1997). Werner syndrome (WS) is the hallmark premature aging disorder in which affected humans appear older than their chronological age. The protein WRNp, defective in WS, has helicase function, DNA-dependent ATPase, and exonuclease activity. Although WRNp functions in nucleic acid metabolism, there is little or no information about the pathways or protein interactions in which it participates. Here we identify Ku70 and Ku86 as proteins that interact with WRNp. Although Ku proteins had no effect on ATPase or helicase activity, they strongly stimulated specific exonuclease activity. These results suggest that WRNp and the Ku complex participate in a common DNA metabolic pathway.

Results

The carboxyl terminus of WRNp interacts physically with the Ku86/70 complex

The exonuclease domain of WRNp is located in the amino-terminal end of the protein and the helicase/ATPase domain in its central part (Fig. 1A). Because the carboxyl terminus [C-WRNp] of WRNp lacks homology to known functional domains, we searched for interactions with this portion of the protein. The carboxyl terminus was cloned into an Escherichia coli expression vector and the recombinant protein was expressed and purified (Fig. 1B). This fragment was immobilized on an agarose bead matrix, and specific proteins were identified by chromatographing HeLa nuclear extract through the column. Columns either without protein attached or with excess β-galactosidase or matrix alone column (Fig. 3B), the Western blot (Fig. 2). This result suggests dimerization or a higher order assembly of WRNp, an observation that has not been reported previously.

Two strong bands [Fig. 3A, arrows] were observed in the 0.2-M salt eluate at ~70 and 90 kD, which were not present in the eluate from the β-galactosidase or matrix columns. These bands were excised and identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS) to be the Ku70 and Ku86 proteins. The identities of the Ku proteins were verified by Western blot [Fig. 3B] using antibodies against Ku70 and Ku86. As there was no binding of Ku70 or Ku86 to either the β-galactosidase or matrix alone column [Fig. 3B], the C-WRN interactions with Ku proteins appears to be specific. Fluorimager analysis demonstrated that the recombinant C-WRNp and Ku proteins contained no DNA, ruling out that the interactions were mediated via DNA (data not shown).

To confirm this interaction in vivo and to investigate whether the Ku proteins bind to full-length WRNp, an antibody against the amino terminus of WRNp was gen-

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Coomassie staining of C-WRNp.

The central region contains the conserved 7 motif helicase domain. (C-WRNp fragment. The amino terminus contains the 3 conserved exonuclease motifs.

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ATPase activity (DNA effector (Orren et al. 1999; Brosh et al. 1999). The DNA-dependent ATPase that prefers ssDNA as the substrate was shown to be an exonuclease with preference for 3'→5' or 5'→3' directionality. Our studies agree with the notion that WRNp is a 3'→5' exoribonuclease with preference for 3' recessed ends (Fig. 5; D. Orren, unpubl.). To examine the effect of Ku86/70 on the exonuclease activity of WRNp, a DNA substrate with one blunt end and one recessed 3' end was designed (Fig. 5B) that is not detectably unwound by the helicase activity of WRNp (data not shown). Exonuclease digestion of this substrate by WRNp in the presence or absence of Ku86/70 is shown in Figure 5C. Ku86/70 alone does not have nuclease activity on this substrate. At low concentrations of WRNp alone, digestion from the 3' recessed end is limited. At higher concentrations of WRNp, there is extensive 3'→5' exonuclease activity (Fig. 5C, lane 8, 120 fmoles). When Ku86/70 is added with WRNp, the extent of digestion into the substrate is increased dramatically (Fig. 5C,D). Heat inactivation of Ku86/70 completely abolishes this stimulatory effect (Fig. 5C). Moreover, a WRNp with a mutation at a conserved amino acid (E84A) in its nuclease domain (Huang et al. 1998) has no detectable 3'→5' exonuclease activity whether Ku86/70 is present or not (Fig. 5C). These results show that addition of Ku86/70 stimulates the 3'→5' exonuclease activity of WRNp. The stimulation of wild-type WRNp exonuclease activity occurs over a broad range of Ku86/70 concentrations, and appears to be optimal at approximately equimolar ratios (Fig. 5E). In contrast, the addition of Ku86/70 does not detectably stimulate the 3'→5' exonuclease activities of other exonucleases, prokaryotic exonuclease III or the Klenow fragment of DNA polymerase I (Fig. 5F). Thus, this stimulation of exonuclease activity is specific for WRNp.

The Ku complex has been reported to have some DNA unwinding activity (Tuteja et al. 1994, Yoo and Dynan 1998), although other studies find no unwinding (Tuteja

Figure 1. Purification of the carboxyl terminus of Werner protein. (A) Schematic of C-WRNp fragment. The amino terminus contains the conserved 7 motif helicase domain. (B) SDS-PAGE with Coomassie staining of C-WRNp.

Ku complex has no effect on WRNp helicase or ATPase activities

Next, we investigated whether the physical interaction between WRNp and the Ku86/70 complex might mediate a change in WRNp activity. WRNp is a processive DNA-dependent ATPase that prefers ssDNA as the DNA effector (Orren et al. 1999; Brosh et al. 1999). The ATPase activity \( k_{\text{cat}} \) of WRNp [7 nM] in the absence of Ku86/70 was determined to be 139/min, consistent with previously published data (Orren et al. 1999; Brosh et al. 1999). In control reactions, Ku86/70 (70 nM, heterotrimer) alone did not demonstrate any detectable ATP hydrolysis over background. When Ku86/70 and WRNp were preincubated together with M13 ssDNA and reactions were initiated with \([^{3}H]ATP\), the \( k_{\text{cat}} \) for ATP hydrolysis by WRNp was 152/min, indicating that Ku protein does not significantly modulate ATP hydrolysis catalyzed by WRNp.

The helicase activity of WRNp was examined on a M13 28-bp partial duplex DNA substrate in the presence or absence of Ku86/70 [Fig. 5A]. Reaction products were analyzed by nondenaturing gel electrophoresis. WRNp (15 nM monomer) alone unwound ~70% of the helicase substrate in a 30-min reaction [Fig. 5A, lane 1], whereas Ku86/70 (128 nM) alone did not detectably unwind the double-stranded (ds) DNA substrate [Fig. 5A, lane 5]. When WRNp and Ku86/70 were preincubated together with the dsDNA substrate and reactions were initiated with ATP, Ku86/70 (8–128 nM) had no effect on WRNp helicase activity (Fig. 5A, lanes 2–6). Moreover, Ku86/70 (8–128 nM) did not stimulate WRNp helicase activity on the 28-bp partial duplex substrate with an amount of WRNp (3 nM monomer) that unwinds only 5% of the double-stranded DNA substrate [A. Machwe and D. Orren, unpubl.]. These results demonstrate that Ku86/70 neither stimulates nor inhibits WRN helicase activity on short DNA duplexes.

Figure 2. Specific proteins bound to C-WRNp. C-WRNp binds to full-length WRNp (~160 kD). Western blot with anti-WRNp antibody. [Lane 1] Nuclear extract, positive control; [lane 2] β-galactosidase column eluate, [lane 3] C-WRNp column eluate.
et al. 1994). We did not observe any unwinding of a M13 partial duplex substrate by Ku86/70 [Fig. 5A] or of the dsDNA substrate [Fig. 5B] used for exonuclease studies [data not shown]. A possible source of this controversy might arise from its tight binding to WRNp, which could result in copurification of WRNp in Ku preparations.

The possibility existed that Ku86/70, in the presence of ATP, caused a local unwinding of the substrate that would then stimulate the exonuclease activity of WRNp. To test this possibility, we performed an exonuclease assay in the absence of ATP [Fig. 5F]. Ku86/70 still greatly stimulates the WRN exonuclease activity under those conditions. This stimulation is also observed in a WRNp K mutant, devoid of helicase activity [Brosh et al. 1999] [Fig. 5G, lanes 5,6]. These results rule out the possibility that either WRNp or Ku unwinding activity is involved in the stimulation of WRN exonuclease.

Discussion

We have shown that the carboxyl terminus of the WRNp interacts strongly with the Ku86/70 complex. This physical interaction was also observed in cell extracts and between the purified proteins. We ascertained that no DNA was present under these conditions, thus excluding the hypothesis that this interaction could occur via a DNA bridge. The Ku86/70 complex strongly stimulated the exonuclease activity of WRNp, although it had no effect on its DNA-dependent ATPase or helicase activity. This stimulatory effect is mediated by the interaction between WRNp and Ku86/70 and serves to dramatically enhance the processivity of the exonuclease function of WRNp. The functional effect of this interaction occurs at the amino terminus, where the exonuclease activity is located. It is possible that there are other binding sites between Ku86/70 and WRNp. The interaction with Ku stimulates the exonuclease activity of WRNp via a direct molecular binding without any local unwinding of DNA by the Ku86/70 complex.

Several studies have shown that Ku86/70 binds to DNA ends and other types of discontinuity in dsDNA [Smith and Jackson 1999]. It is a tightly associated heterodimer that, together with the ~470-kD catalytic subunit, DNA–PKcs, form the DNA-dependent protein kinase [Featherstone and Jackson 1999]. This complex is involved in repairing DNA double-strand breaks [DSBs] formed during V(D)J recombination or caused, for example, by endogenous oxidative byproducts, ionizing radiation, or certain chemotherapeutic drugs. A current model proposes that the complex joins the DNA ends, whereas a helicase unwinds the DNA duplex locally at the break, permitting annealing of the broken strands at sites of microhomology [Smith and Jackson 1999]. The unannealed DNA ends then might be trimmed by an exonuclease before gap-filling and ligation by a polymerase and ligase [Yoo and Dynan 1998]. No specific helicase or exonuclease that is involved in this process in mammalian cells has as yet been identified. Such exonuclease activity could be performed by WRNp, and the WRNp helicase activity may also participate in the process. WRNp and Ku could act together in recombination and/or DSB repair pathways. Our results suggest that Ku might help direct and stimulate WRN exonuclease activity on DNA ends.
There is good evidence for a role of the WRNp in DNA replication. A prolonged S phase has been observed in WS cells (Poot et al. 1992). The WRNp is 66% homologous to the *Xenopus laevis* FFA1 protein (foci-forming activity 1), which is essential for the formation of replication origins and RPA aggregation (Yan et al. 1998). We have reported previously that WRNp interacts physically and functionally with RPA (Brosh et al. 1999). Furthermore, the WRNp is a part of the replication complex (Lebel et al. 1999). The Ku86/70 and DNA–PK complex is also involved in DNA replication (Ruiz et al. 1999), and thus the main interaction between the Ku complex and WRNp may be via both of their roles in the replication process.

WS cells are not generally hypersensitive to DNA damaging agents. They are, however, sensitive to the carcinogen 4NQO (Gebhart et al. 1988) and to certain topoisomerase inhibitors such as the topoisomerase I inhibitor, camptothecin (Lebel and Leder 1998). One study reports a repair deficiency for higher dose X-ray-induced damage (Weirich-Schwaiger et al. 1994), but others do not. The genomic instability in WS cells (Martin 1997)

Figure 5. WRN exonuclease, but not helicase, is stimulated by Ku86/70. (A) WRNp (15 nM monomer) was incubated with the 28-bp partial duplex in the presence of the indicated concentrations of Ku86/70 under standard helicase reactions (Brosh et al. 1999). Lanes show increasing concentrations of Ku86/70. [Lane 7] No WRNp added, shows that Ku proteins do not unwind this substrate. (B) Heat-denatured control. (C) The exonuclease substrate containing a 5' overhang. (D) Heat denaturation of WRN exonuclease at different concentrations of WRN [fmoles]; Ku was 64 fmoles [10 ng]. (D) Heat denaturation of Ku86/70. (Exo-) Mutant WRNp with no exonuclease activity. (D) Scans of lanes 5 and 6. (E) Ku86/70 stimulation of WRN exonuclease [at 45 fmoles], Ku titration. (F) Ku does not stimulate Exonuclease III or Klenow. (G) Ku stimulates WRN [wild-type and helicase mutant |Brosh et al. 1999|] exonuclease [both at 120 fmoles] in the absence of ATP.

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and the high frequency of deletions and translocations could be compatible with a DSB repair defect. We are currently investigating this in more detail, but as no cell lines with functional domain knockout in the WRN gene are yet available, many primary WS cell lines need to be analyzed.

If WRNp and Ku86/70 act in the same pathway, deficiencies in either component would be expected to have similar biochemical and cellular phenotypes. This notion is supported by a recent characterization of the Ku86 knockout mouse. The mouse has a senescent phenotype and, like WS patients, shows early onset of many age-related changes and a shortened life span [Vogel et al. 1999]. Furthermore, cells deficient in WRNp, Ku70, or Ku86 undergo premature replicative senescence [Gen et al. 1997] and have elevated levels of chromosomal abnormalities. These similarities combined with our experimental results suggest that WRNp and Ku86/70 function in a common pathway, most likely in replication, recombination, or DSB repair. Our data might argue that a defective exonuclease function could lead to an aging phenotype.

Materials and methods

MALDI-MS

The matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed by the HMI/Keck facility at Yale University, where information is posted on the website: http://info.med.yale.edu/wmkehr.

Preparation of WRNp antibodies

Purified denatured N-WRN (100 µg) was injected intraperitoneal on day 0, and booster injections were administered at day 21 and day 28. Serum was collected from tail bleeds and used for immunoprecipitation.

Purification of WRNp and Ku complex

Recombinant human Ku was expressed in insect cells using baculoviral constructs and 70- and 86-kD subunits were separated by PCR from cDNAs (a gift of D. Capra, University of Washington School of Medicine, Seattle). The 70-kD subunit was modified such that the amino acid sequence LEIEGRHHHHHH was placed in front of the stop codon at the carboxyl terminus. Both coding sequences were placed in pFASTBAC1, an E. coli vector, where information is posted on the website: http://info.med.yale.edu/wmkehr.

Ku complex stimulates the Werner protein

Immuno precipitation assays

Immuno precipitation of WRNp was performed in IP buffer (50 mM HEPES at pH 7.4, 0.1 M NaCl, 0.05% Triton X-100, 1 mM EDTA, 1 mM Na vanadate, 1 mM NaF, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin). Nuclear extract [20 mg] prepared from HeLa cells was sequentially incubated for 1 hr at 4°C with the no protein column, the β-galactosidase column, and the C-WRNp column, respectively. Proteins adsorbed to the columns were washed with 10 column volumes of binding buffer to remove weakly bound proteins. The columns were then eluted with binding buffer containing 0.2 M NaCl and 1 M NaCl to identify tight binding proteins. The eluted fractions were dialyzed and concentrated by membrane filtration [Amicon], lyophilized, and samples were analyzed by SDS-PAGE [%] followed by either Coomassie staining or Western blot. All columns were prepared fresh for each experiment and all results were duplicated.

ATPase assay

ATPase assay reaction mixtures [20 µl] contained 40 mM Tris (pH 7.4), 4 mM MgCl2, 5 mM DTT, M13mp18 ssDNA (30 µm nucleotide phosphate), 0.8 mM 32P[ATP (42 cpm/pmol), WRN protein [7 µM, monomer] and/or Ku protein [70 nM, heterodimer]. WRN and Ku proteins were preincubated for 2 min at 24°C in ATPase reaction buffer containing M13 ssDNA effector. Reactions were initiated by the addition of [32P]ATP and incubated at 24°C for 10 min. Samples [5 µl] were removed and evaluated by TLC as described previously [Matson and Richardson 1983]. Less than 20% of the substrate ATP was consumed in the reaction over the entire time course of the experiment.

Helicase assay

Helicase assay reaction mixtures [20 µl] contained 40 mM Tris (pH 7.4), 4 mM MgCl2, 5 mM DTT, 2 mM ATP, WRN helicase [15 µM, monomer], and the indicated amount of Ku86/70 protein. The concentration of the
Klenow exonuclease assays were carried out in 50 mM Tris (pH 7.4), 10 mM MgCl₂, and 1 mM DTT. Reaction tubes were initiated with the addition of ATP and incubated at 24°C for 30 min. Reactions were terminated by the addition of 10 µl of 50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol. The products of helicase were resolved on a 12% nondenaturing polyacrylamide gel. Radiolabeled DNA species in polyacrylamide gels were visualized by a PhosphorImager (Molecular Dynamics). Quantitation of a dsDNA substrate with one blunt end and one 3'-recessed (5'-overhang) end (Fig. 5B), labeled 32-mers were mixed with a twofold excess to 25°C. The annealed dsDNA was then separated from unannealed and excess single-stranded oligonucleotides by nondenaturing polyacrylamide gel electrophoresis. Intact dsDNA substrates were recovered using a QaeX II gel extraction kit (Qiagen) and stored at 4°C. WRNp exonuclease assays were carried out in 50 mM Tris (pH 7.4), 10 mM MgCl₂, and 1 mM DTT. Exo III reactions were done in 67 mM Tris (pH 7.4), 0.66 mM MgCl₂, and 1 mM β-mercaptoethanol. DNA substrates (3 fmol/reaction) were incubated with Klenow (0.2–1.0 units), Exo III (0.005–0.025 units), WRNp (45–120 fmoles), or WRNp-exo− (180 fmoles) and, where indicated, with Ku70/Ku86 complex (12.8–1280 fmoles) for 1 hr at 37°C. The reactions [1 µl volume] were stopped by addition of an equal volume of formamide loading buffer (80% formamide, 0.5× TBE, glycerol, 0.9% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol). The digestion products of these reactions were separated on 15% denaturing polyacrylamide gels and visualized with a PhosphorImager [Molecular Dynamics]. Quantitative comparison of lane lines and generation of line graphs was accomplished using ImageQuant software.

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