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A conserved and species-specific functional interaction between the Werner syndrome-like exonuclease \textit{at}WEX and the Ku heterodimer in \textit{Arabidopsis}

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ABSTRACT

Werner syndrome is associated with mutations in the DNA helicase RecQ3 [a.k.a. \textit{Homo sapiens} (hs)-WRN]. The function of \textit{hs}WRN is unknown although biochemical studies suggest a role in DNA ends stability and repair. Unlike other RecQ family members, \textit{hs}WRN possesses an N-terminal domain with exonuclease activity, which is stimulated by interaction with the Ku heterodimer. While this interaction is intriguing, we do not know whether it is important for \textit{hs}WRN function. Although flies, worms, fungi and plants do not have RecQ-like (RQL) helicases with an intrinsic exonuclease activity, they possess proteins having domains homologous to the \textit{hs}WRN exonuclease. The genome of \textit{Arabidopsis thaliana} (\textit{at}) encodes multiple RQL and a single protein with homology to the WRN exonuclease domain, \textit{at}WEX (Werner-like Exonuclease). Here we show that \textit{at}WEX has properties that are similar to \textit{hs}WRN. \textit{at}WEX binds to and is stimulated by \textit{at}Ku. Interestingly, stimulation by Ku is species-specific, as \textit{hs}Ku does not stimulate \textit{at}WEX exonuclease activity. Likewise, \textit{at}Ku fails to enhance the exonuclease activity of \textit{hs}WRN. Thus, in spite of the differences in structural organization, the functional interaction between WRN-like exonucleases and Ku has been preserved through evolutionary radiation of species, emphasizing the importance of this interaction in cell function.

INTRODUCTION

Werner syndrome (WS), a human disease with many features of premature aging, is caused by mutations within a single gene located on human chromosome 8. The disease becomes evident in late adolescence and involves the frequent occurrence of conditions generally observed during normal aging, such as atherosclerosis, osteoporosis, type II diabetes mellitus, myocardial infarction and cancer (1). Cells isolated from WS patients display a shorter replicative life span (2) and genomic instability characterized by an elevated rate of chromosomal translocations and extensive genomic deletions (3). The gene mutated in WS encodes a protein [\textit{Homo sapiens} (hs)RecQ3 or WS protein—\textit{hs}WRN] that is a member of the RecQ family of helicases (4). In contrast to other RecQ helicases, \textit{hs}WRN possesses an N-terminal domain with exonuclease activity. The presence of exonuclease and helicase activities suggests that WRN is involved in a nucleic acid reaction; however, the precise cellular function of this protein remains poorly defined. During the last few years, a growing number of biochemical studies have reported the identification of proteins that interact with \textit{hs}WRN (5–7). Work from our and another laboratory has shown that \textit{hs}WRN binds to the Ku70/80 heterodimer (Ku) (8,9). Remarkably, our studies showed that Ku recruits \textit{hs}WRN to DNA ends and alters the properties of \textit{hs}WRN exonuclease activity (8,10). Ku is a factor that binds to DNA ends and is involved in the repair of double-strand DNA breaks by non-homologous DNA end joining (NHEJ) (11). Moreover, Ku is also found at telomeric ends (12), suggesting that this factor may, depending on the context, stimulate or inhibit ligation of DNA ends. How these distinct functions are regulated is currently unknown, but it is possible that specific protein interactions play a role in.
determining the fate of DNA ends bound to Ku. It remains to be determined whether the interaction between hsWRN and Ku is important for some aspects of DNA ends metabolism or possibly other cellular processes. One way to assess the biological relevance of the interaction between these proteins is to test whether it is conserved across phylogenetically distant taxa. While the bifunctional structure of hsWRN with both an exonuclease and a helicase is conserved between human and mice, there are no known RecQ-like (RQL) helicases with an intrinsic exonuclease activity in flies, worms, fungi or plants. Nevertheless, genes in the Arabidopsis genome encode two types of proteins with high homology to WRN protein: multiple RQL and a single exoC-domain exonuclease (Werner-like Exonuclease, atWEX) (13) (Figure 1). Biochemical studies have shown that atWEX has 3′–5′ exonuclease activity (14) and yeast-two-hybrid assays have indicated that it binds to RQL2, which among the RQLs is phylogenetically closer to hsWRN (13). Moreover, a study of an Arabidopsis T-DNA insertion mutant line with reduced atWEX mRNA expression showed that atWEX is required for post-transcriptional gene silencing (15), an epigenetic mechanism of gene expression regulation related to RNA interference. Interestingly, atWEX is homologous to Caenorhabditis elegans mut7; genetic analysis of mutated worm strains indicated that mut7 gene product is required for transposon silencing and RNA interference (16). However, the analysis of mice with a null mutation in the WRN homologue gene failed to show any involvement of this protein in the RNA interference pathway (17).

In this study, we characterize the properties of atWEX and examine its relationship to atKu. We demonstrate that atWEX physically interacts with atKu and atKu. Moreover, we present data showing that atWEX exonuclease activity is stimulated by atKu but not by hsKu. Furthermore, we present data showing that atWEX exonuclease is stimulated by hsKu but not by its plant counterpart. Thus, we conclude that Ku–WRN interaction is conserved and specific.

MATERIALS AND METHODS
Cloning of atWEX, atKu70 and atKu80

RNA was extracted using the Trizol method (Invitrogen) from flower buds of Arabidopsis thaliana accession Columbia (Col-0) er105 for wild-type atWEX, from the mutagenized derivative of Col-0 er105 for wex-2, and from Col-0 (for atKu70 and atKu80). A first strand cDNA segment containing the complete open reading frame of the target genes was synthesized by RT–PCR using RT–MMLV and random hexamers according to established protocols. The following primers for each gene were designed with NdeI sites (underlined) adjacent to the start ATG using Primer3: atWEX, 5′-CCAGAGGATCCAGCTGGAAGAACG-3′; atKu70, 5′-CCAGAGGATCCACAGCATAGACG-3′; and atKu80, 5′-CCAGAGGATCCACGGCATAGACG-3′. The resulting products were cloned into the expression vector pEGFP-N2 (Clontech) and sequenced.
Expression and purification of recombinant proteins
Flag-tagged at WEX, HA-at Ku80, His-at Ku80, myc-at Ku70 and at Ku70 were expressed individually or in various combinations in sf9 cells using a baculovirus expression system. The cDNAs coding for these factors were cloned into a pVL-based vector, and were then cotransfected with linearized BaculoGold DNA (Pharmingen) into sf9 cells to generate the recombinant baculoviruses. Cells infected with the recombinant baculovirus expressing FlagWEX were lysed in Lysis Buffer (10 mM Tris, pH 7.9, 100 mM KCl, 1.5 mM MgCl2 and 5 mg/ml aprotinin), and incubated on ice for 20–30 min. The samples were then resolved by electrophoresis through a 4% polyacrylamide gel at 10 V/cm in the cold room.

Protein interaction assays
Sf9 cells infected with the appropriate recombinant baculoviruses were harvested 42–48 h postinfection and washed with 1x phosphate-buffered saline. Cell pellets were resuspended in NTN containing a cocktail of protease inhibitors (1 mM PMSF, 1 mg/ml pepstatin A, 5 mg/ml leupeptin and 5mg/ml aprotinin). Baculovirus amplification and sf9 cells were maintained as described in (19). Recombinant hsWRN and Ku were purified from baculovirus-infected sf9 cells as described previously (8).

Exonuclease assay
Exonuclease activity was measured with the following DNA substrates: 20-oligomer A1 (5'-CGTACGAGATTATTATCGAGC-3'), 20-oligomer A2 (5'-GCTGAGAATATTAGCTAG-3') complementary to A1, and 46-oligomer A3 (5'GCGG-GGAAGTTGGCTTCAGGAGAGTTT-3') partially complementary to A1. Oligonucleotides were labeled at the 5' end with radiolabeled ATP. The appropriate oligonucleotides were annealed by boiling and slow cooling to room temperature. Reaction mixtures contained 40 mM Tris–HCl (pH 7.5), 4 mM MgCl2, 5 mM DTT, 1 mM ATP, 0.1 mg/ml BSA, DNA substrates (~40 fmol), 100,000 c.p.m.), and 50–200 fmol of atWEX protein, 100 fmol of WRN, 50–200 fmol of atKu or hsKu in a final volume of 10 µl. The reaction mixtures were incubated at room temperature for 10–20 min and then the reactions were terminated by the addition of 2 µl of a formamide-dye solution (95% formamide, 50 mM EDTA, 0.5% bromphenol blue and 0.5% xylene cyanol). After incubation at 95°C for 3 min, DNA products were resolved by either 12 or 16% polyacrylamide-urea gel electrophoresis and visualized by autoradiography.

Electrophoretic mobility shift assay
The 20mer (A1) oligonucleotide was labeled at the 5' end with radiolabeled ATP and T4 polynucleotide kinase and then annealed to a partially complementary 46mer (A3). Radiolabeled oligonucleotide (80 fmol and 200,000 c.p.m.) was incubated with increasing amounts (100–400 fmol) of atKu in 10 µl of buffer (10 mM Tris–HCl, pH 7.5, 80 mM NaCl, 4 mM KCl, 2 mM EDTA and 10% glycerol) at 25°C for 10 min. The samples were then resolved by electrophoresis through a 4% polyacrylamide gel at 10 V/cm in the cold room. The gels were dried on Whatman 3MM paper and subjected to autoradiography.

RESULTS
We wanted to examine at WEX exonuclease activity and its relationship to hsWRN and Ku. For this purpose, we isolated cDNAs for at WEX, at Ku70 and at Ku80 and cloned them in frame with a flag-epitope tag into baculovirus expression vectors. Each protein was expressed and purified from insect cells by affinity purification (Figure 2A). To demonstrate that they possess their respective activities, each recombinant protein was first tested in biochemical assays. Recombinant at Ku was examined by electrophoretic mobility shift assays for its ability to bind to DNA. As shown in Figure 2B, the addition of atKu to a reaction mixture containing a radiolabeled double-stranded DNA (dsDNA) oligomer resulted in the formation of a strong protein–DNA complex, indicating that atKu, like the homologous human factor (8), binds to linear dsDNA molecules. We then examined the exonuclease activity of the recombinant at WEX. Incubation of at WEX with radiolabeled, dsDNA substrates produced strong hydrolysis of 3' recessed ends (Figure 2C, lanes 2–4), and somewhat less efficient hydrolysis of 3' overhang or blunt ends (Figure 2A, lanes 7–9, and Figure 2B, lanes 2–4). A mutant atWEX (encoded by the wex-2 allele) missing the conserved exonuclease domain III did not show any hydrolytic activity (Figure 2A, lanes 5 and 10, Figure 2B, lane 5), indicating that the observed activity

5'-CCATATGTCATCGTCAAATTGGATCGACGAC-3' and 5'-GAAGCCTCTGACAGCATCGCAAGAA-3'; atKu70, 5'-CA-
TATGAAATTGGAGCCAGATGAG-3' and 5'-CCATITCC-
CCTCAAAAAACAGACA-3'; atKu80, 5'-CATATGGGCA-
GAATGGAGGAGTT-3' and 5'-TGTAGGGTCTCC-
GAGATTGAATCTTT-3'. PCR products were cloned using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was then prepared using a plasmid extraction kit (Qiagen) and sequenced by the Big-Dye fluorescent chain-terminator method. The sequence was analyzed using Sequencher (v4.1.2). The wexW allele (W226*) of the atWEX gene (At4g13870) was identified in a TILLING screen (18). This mutation disrupts a restriction site for the enzyme NlaIV, facilitating genotyping.

Cell lysates were cleared by centrifugation at 18,000 g for 5 min and then incubated with the appropriate

Electrophoretic mobility shift assay
The 20mer (A1) oligonucleotide was labeled at the 5' end with radiolabeled ATP and T4 polynucleotide kinase and then annealed to a partially complementary 46mer (A3). Radiolabeled oligonucleotide (80 fmol and 200,000 c.p.m.) was incubated with increasing amounts (100–400 fmol) of atKu in 10 µl of buffer (10 mM Tris–HCl, pH 7.5, 80 mM NaCl, 4 mM KCl, 2 mM EDTA and 10% glycerol) at 25°C for 10 min. The samples were then resolved by electrophoresis through a 4% polyacrylamide gel at 10 V/cm in the cold room. The gels were dried on Whatman 3MM paper and subjected to autoradiography.
was intrinsic to \textit{at} WEX and not caused by a co-purifying contaminant.

Next, we determined whether \textit{at} WEX binds to \textit{at} Ku. For this purpose, \textit{Sf9} insect cells were infected with various combinations of recombinant baculoviruses expressing epitope-tagged \textit{at} WEX, \textit{at} Ku80 and \textit{at} Ku70. Extracts from the infected cells were incubated with the appropriate antibody resin to capture flag-\textit{at} WEX (Figure 3A) or myc-\textit{at} Ku70 (Figure 3B), and the resulting immunoprecipitated products were resolved by SDS–PAGE. The presence of \textit{at} Ku80 in the flag-\textit{at} WEX immunoprecipitation reaction, or \textit{at} WEX in the myc-\textit{at} Ku70 immunoprecipitation reaction, was monitored by western blot with HA and flag antibodies, respectively. The results of these experiments indicate that both \textit{at} Ku70 and \textit{at} Ku80, as a complex or as single subunits, co-immunoprecipitated with \textit{at} WEX. Reciprocal immunoprecipitation reactions confirmed that \textit{at} WEX bound to Ku through interactions with both subunits (data not shown). The overall identity between \textit{H. sapiens} and \textit{Arabidopsis} Ku70 and 80 is 48 and 43\%, respectively (BLASTP 2.2.10 analysis). Notwithstanding this limited sequence identity, \textit{at} Wex also binds to \textit{hs} Ku (Figure 3C), suggesting conservation in the interaction domains between these two factors. The possibility that this interaction was mediated by tethering of the proteins to DNA was discounted by treatment of the extracts with DNAseI (Figure 3C). In conclusion, these results indicate that \textit{at} WEX, as observed with \textit{hs} WRN, binds to Ku.

We next examined whether \textit{at} Ku influences the exonuclease activity of \textit{at} WEX. To this end, purified recombinant proteins were incubated with radiolabeled dsDNA substrates and the products of the reactions were examined by denaturing gel electrophoresis and autoradiography. As shown in Figure 4A and B (lanes 2–4), the addition of \textit{at} Ku to the reaction mixture resulted in increased hydrolysis of 3’ recessed and blunt ended DNA substrates, indicating that \textit{at} Ku stimulates the nucleolytic activity of \textit{at} WEX, as shown previously for \textit{hs} WRN and \textit{hs} Ku (8). Stimulation of \textit{at} WEX activity by \textit{at} Ku was also observed on double-stranded oligomers with a 3’ overhang.

Figure 2. Activities of recombinant \textit{at} WEX and \textit{at} Ku. (A) Affinity-purified \textit{at} Ku (lane 1), \textit{at} WEX (lane 2) and mutant \textit{at} WEXm (\textit{wex}-2) (lane 3). Proteins were resolved by SDS–PAGE and visualized by silver staining. (B) \textit{at} Ku binds to dsDNA. Radiolabeled 20mer (A1)/46mer (A3) DNA substrate was incubated with increasing amounts of \textit{hs} Ku for 10 min at room temperature. The reactions were analyzed by 4% native PAGE, and the DNA–protein complexes were visualized by autoradiography (lanes 1, DNA probe only; lanes 2–4, 100, 200, 300 fmol of \textit{at} Ku, respectively). (C) The 3’–5’ exonuclease activity of \textit{at} WEX. Purified wild-type or mutant (\textit{at} WEXm; \textit{W266}*; \textit{at} WEX) \textit{at} WEX were incubated with 3’ recessed, radiolabeled 20mer (A1)/46mer (A3) or blunt radiolabeled 20mer (A1)/20mer (A2) DNA substrate at room temperature for 20 min. Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 1, 20mer/46 mer probe only; lane 2–4, 50, 100 and 200 fmol of \textit{at} WEX; lane 5, 100 fmol of \textit{at} WEXm; lane 6, 20mer/20 mer probe only; lane 7–9, 50, 100 and 200 fmol of \textit{at} WEX, respectively; lane 10, 100 fmol of \textit{at} WEXm). (D) Purified \textit{at} WEX and \textit{at} WEXm were incubated with a radiolabeled 3’ protruding 46mer (A3)/20mer (A1) DNA substrate at room temperature for 20 min. Products were analyzed by 12% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 46mer/20mer probe only; lanes 2–4, 400, 500 and 600 fmol of \textit{at} WEX, respectively; lane 5, 500 fmol of \textit{at} WEXm).
Cleared lysates were divided into two aliquots and incubated at 25°C for 2 h at 4°C on a nutator. The resin was washed extensively and then boiled in SDS sample buffer to release the bound proteins, which were separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. Blotted at Ku80 was detected with HA antibody. The lower panel shows the expression level of at Ku80 in the cell extract from each coinfection. (B) Sf9 cells were infected with baculoviruses expressing Flag-at WEX (lane 1), myc-at Ku70, His-at Ku80 and Flag-at WEX (lane 2), or myc-at Ku70 and Flag-at WEX (lane 3). Cells were harvested 48 h postinfection, lysed in NTN buffer and the cleared lysates were incubated with anti-Flag sepharose beads for 2 h at 4°C on a nutator. Beads were then washed extensively and boiled in SDS sample buffer to release the bound proteins, which were separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. Blotted at Ku70 in the cell extract from each coinfection. (C) Sf9 cells were infected with baculoviruses expressing His-hsKu70, hsKu80 and Flag-at WEX (lane 1), hsKu80 and Flag-at WEX (lane 2), His-hsKu70 and Flag-at WEX (lane 3), or hsKu80 and His-hsKu70 alone (lane 4). Cells harvested 48 h postinfection were lysed in NTN buffer. Cleared lysates were divided into two aliquots and incubated at 25°C for 15 min in the absence (upper panel) or presence (lower panel) of DNase I (0.5 U/µl). Extracts were centrifuged briefly and incubated with anti-Flag sepharose beads for 2 h at 4°C on a nutator. Beads were then washed extensively and boiled in SDS sample buffer to release the bound proteins, separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. The presence of hsKu80 and hsKu70 in the immunoprecipitation products was detected with anti-Ku70 and Ku80 antibodies (Santa Cruz Biotechnology). Lower panel shows the expression level of hsKu80 and hsKu70 in the protein extract from each coinfection.

**DISCUSSION**

Rapid progress in genome analysis is leading to the identification of the genes responsible for many human diseases. Understanding the molecular basis of these inherited conditions, however, hinges on uncovering the function of the proteins encoded. Such an endeavor can be challenging, as in the case of the WS protein (WRN). WRN is a protein that may play an important role in human aging. Loss-of-function mutations in the WRN gene are associated with the premature onset of degenerative conditions generally observed during normal aging, such as cataracts, graying and loss of hair, atherosclerosis, loss of subcutaneous fat, diabetes, osteoporosis and certain types of cancer. WRN has been implicated in pathways such as DNA repair, recombination and replication, but the precise cellular function of WRN, and the molecular basis of WS, remains to be determined.

An important clue of function is provided by the identification of physical interactions between the protein of interest and other cellular factors. In previous studies we showed that the heterodimeric factor Ku forms a stable complex with WRN and stimulates its exonuclease activity. Ku is required for the repair of DNA DSBs by NHEJ (11), but has also been...
implicated in the maintenance of telomere structure, acting as negative regulator of telomerase in animals (20) and plants (21). In plants, atKu is required for proper maintenance of the telomeric C strand and regulates the extension of the telomeric G strand (22). In contrast to the mammalian orthologous complex (20,23), however, atKu is not required for fusion of critically short telomeres in telomerase-deficient Arabidopsis. Understanding the physiological role of the Ku–WRN interaction may provide key insights on WRN function. The evolutionary history of this interaction should help to elucidate its significance. atWEX is an Arabidopsis protein with homology to H. sapiens WRN. The similarity between atWEX and the N-terminal exonuclease domain of WRN prompted us to ask whether there was a functional relationship between these two factors. Conservation of the interaction across distant taxa would underscore its importance to the function of WRN. Our data show that recombinant atWEX has intrinsic exonuclease activity that hydrolyzes dsDNA oligomers with 3’ overhang, blunt and 3’ recessed ends. These results differ from a previous report, which showed that atWEX does not hydrolyze DNA with blunt ends (14). This discrepancy is probably owing to the different expression systems used for the isolation of recombinant proteins for the exonuclease assays [bacteria (14) versus insect cells (this study)]. The results of our studies also indicate that the exonuclease activity of atWEX only partially resembles the corresponding activity of hsWRN, which hydrolyzes dsDNA oligomers with a 3’ recessed end and is inactive on DNA with a 3’ overhang or blunt ends (8). Although atWEX displays broader substrate specificity, we cannot rule out that this difference results from the presence of the RecQ helicase and other domains within the C-terminal region of hsWRN, as these may influence the exonuclease activity of hsWRN. Indeed, a mutant hsWRN comprising only the exonuclease domain, hsWRN(1–388), displays stronger exonuclease activity than full-length hsWRN [B. Li and Lucio Comai, unpublished data (10)]. When we tested whether Ku influences the activity of atWEX, we observed that atWEX exonuclease was strongly stimulated by atKu, but only to a minor degree by hsKu. This species-specific stimulation was also observed in the complementary experiment, which shows that atKu could not augment hsWRN exonuclease activity. The inability of WRN-like exonucleases to be efficiently stimulated by heterologous Ku proteins indicates that Ku stimulation is not the result of a topological constraint on the substrate resulting from the binding of Ku to DNA. Rather, binding of hsWRN orthologs to Ku proteins and stimulation of exonuclease activity is a specific regulatory interaction that predates the split between plants and animals, an event ∼1.5 billion years old, and has been maintained through multiple evolutionary changes of these proteins. Divergence of the functional interaction may indicate that the WRN/WEX–Ku complex has evolved within each species to optimize function. We propose, therefore, that the relationship between WRN exonuclease and Ku is highly significant and that it is informative in determining the function of hsWRN and related proteins.

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