Werner Protein Is a Target of DNA-dependent Protein Kinase *in Vivo* and *in Vitro*, and Its Catalytic Activities Are Regulated by Phosphorylation*

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Human Werner Syndrome is characterized by early onset of aging, elevated chromosomal instability, and a high incidence of cancer. Werner protein (WRN) is a member of the *rec*Q gene family, but unlike other members of the *rec*Q family, it contains a unique $3' \rightarrow 5'$ exonuclease activity. We have reported previously that human Ku heterodimer interacts physically with WRN and functionally stimulates WRN exonuclease activity. Because Ku and DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase (DNA-PK), form a complex at DNA ends, we have now explored the possibility of functional modulation of WRN exonuclease activity by DNA-PK. We find that although DNA-PKcs alone does not affect the WRN exonuclease activity, the additional presence of Ku mediates a marked inhibition of it. The inhibition of WRN exonuclease by DNA-PKcs requires the kinase activity of DNA-PKcs. WRN is a target for DNA-PKcs phosphorylation, and this phosphorylation requires the presence of Ku. We also find that treatment of recombinant WRN with a Ser/Thr phosphatase enhances WRN exonuclease and helicase activities and that WRN catalytic activity can be inhibited by rephosphorylation of WRN with DNA-PK. Thus, the level of phosphorylation of WRN appears to regulate its catalytic activities. WRN forms a complex, both *in vitro* and *in vivo*, with DNA-PKC. WRN is phosphorylated *in vivo* after treatment of cells with DNA-damaging agents in a pathway that requires DNA-PKcs. Thus, WRN protein is a target for DNA-PK phosphorylation *in vitro* and *in vivo*, and this phosphorylation may be a way of regulating its different catalytic activities, possibly in the repair of DNA dsb.

Werner syndrome (WS)[1] is a human autosomal recessive disorder characterized by early onset of premature aging char-acteristics including graying and loss of hair, wrinkling and ulceration of skin, atherosclerosis, osteoporosis, and cataracts. In addition, WS patients exhibit an increased incidence of diabetes mellitus type 2, hypertension, and malignancies (1). The gene (*WRN*), defects in which are responsible for WS, encodes a 1,432-amino acid protein (WRN) (2) that has both $3' \rightarrow 5'$ helicase and $3' \rightarrow 5'$ exonuclease activities (3–7). Although WRN appears to play an important role in DNA metabolism, the precise cellular roles of both the helicase and exonuclease activities of WRN remain to be determined.

Cells from patients with WS show premature replicative senescence compared with cells derived from normal individuals (8). The WS cellular phenotype suggests correlations among faulty DNA metabolism, genomic instability, and senescence. WS cells show hypersensitivity to selected DNA-damaging agents including 4-nitroquinoline-1-oxide (4NQO) (9), topoisomerase inhibitors (10), and certain DNA cross-linking agents (11). Compared with normal cells, WS cells also exhibit increased genomic instability including higher levels of DNA deletions, translocations, and chromosomal breaks (12, 13). These studies suggest that WRN plays an important role in DNA metabolism possibly by participating in DNA repair, replication, and/or recombination pathways.

We have previously identified a physical and functional interaction between WRN and the Ku heterodimer (14). This finding has been confirmed recently by an independent laboratory (15). Together with the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Ku is required for repair of DNA double strand breaks (dsb) generated during recombination and by reactive oxygen species resulting from endogenous metabolism or treatment with ionizing radiation and certain mutagens (16). We have previously shown that Ku significantly stimulates the $3' \rightarrow 5'$ exonuclease activity of WRN (14), suggesting that WRN and Ku could act in a common pathway of DNA metabolism involving the exonuclease function of WRN. This hypothesis is supported by the observation that mice lacking the Ku80 subunit show a premature aging phenotype similar to that of WS patients (17). Furthermore, cells deficient in WRN, Ku70, or Ku80 all show genomic instability and undergo premature replicative senescence (18, 19).

DNA dsb can be created by ionizing irradiation or during V(D)J recombination, a process that generates immunological diversity. One of the major pathways for the repair of dsb involves an end-rejoining reaction that requires DNA-PK.

[1] The abbreviations used are: WS, Werner Syndrome; BSA, bovine serum albumin; DNA-PK, DNA-dependent protein kinase (Ku heterodimer and DNA-PKcs); DNA-PKcs, catalytic subunit of DNA-PK; dsb, double strand break(s); NHEJ, nonhomologous end rejoining; 4NQO, 4 nitroquinoline-1-oxide; PP1, protein phosphatase 1; WRN, Werner protein; ATM, ataxia telangiectasia-mutated protein.

Werner Protein Is Phosphorylated by DNA-PK

DNA-PK is composed of a 465-kDa catalytic subunit (DNA-PKcs) and the Ku70/80 heterodimer (20). The precise role of DNA-PK in DNA dsb repair in mammalian cells is not clear, but both Ku and DNA-PKcs are essential for the processing of DNA ends. Current models predict that Ku binds to ends of double-stranded DNA and then recruits DNA-PKcs to form the active protein kinase complex. DNA-PK is essential for efficient DNA dsb repair because cells lacking either DNA-PKcs or Ku are sensitive to ionizing radiation and defective in V(D)J recombination (20). DNA-PK has been shown to phosphorylate a variety of proteins *in vitro*, including SP1, SV40 T antigen, p53, and replication protein A (20, 21). Although the kinase activity of DNA-PK is required *in vivo* (22), its physiological substrates are not known.

Because WRN has been shown to interact with Ku, we have explored its role in the DNA-PK damage response pathway. In this study we have examined functional interactions among WRN, Ku, and DNA-PKcs. We find that DNA-PKcs, Ku, and WRN interact on a DNA substrate and that DNA-PK down-regulates the exonuclease activity of WRN. Correspondingly, dephosphorylation of WRN increases its helicase and exonuclease activities. We also show that WRN is phosphorylated *in vitro* by DNA-PK and that DNA damage-induced phosphorylation of WRN is absent from a human cell that lacks DNA-PKcs. Our results would suggest that WRN may play a role in DNA-PK-mediated end rejoining.

EXPERIMENTAL PROCEDURES

Proteins

Amplified baculovirus was used to infect Sf9 insect cells for overexpression of WRN protein as described before (7). The protein was purified by DEAE-Sepharose (Amersham Biosciences), Q-Sepharose (Amersham Biosciences), and nickel-nitrilotriacetic acid (Invitrogen) chromatography as described previously (7). Human Ku heterodimer (23) and DNA-PKcs (24) were purified as reported. The purity of these proteins was checked routinely, and they specifically did not contain any ATM protein (data not shown). Baculovirus constructs for hexahistidine-tagged full-length WRN protein were kindly provided by Dr. Mathew Gray (University of Washington, Seattle).

Materials

Wortmannin and LY294002 were from Alexis Biochemicals. 4NQO was from Janssen Chemical. Bleomycin sulfate was from Sigma. T4 polynucleotide kinase was from New England Biolabs. Protein phosphatase 1 (PP1) was from Roche Molecular Biochemicals. $[\gamma^{-32}P]ATP$ was from PerkinElmer Life Sciences. ATP was purchased from Amersham Biosciences.

DNA Substrates

Exonuclease Substrates—Single-stranded DNA oligomers (72-mer and 53-mer) were obtained from Invitrogen. 7 pmol of 53-mer was 5′-labeled with 60 μCi of $[\gamma^{-32}P]ATP$ (3,000 Ci/mmol) and 10 units of polynucleotide kinase using standard conditions. To construct a double-stranded DNA substrate with one blunt end and one 3′-recessed (5′-overhang) end, labeled oligomer was mixed with a 2-fold excess of unlabeled 72-mer, heated together at 90 °C for 5 min, then cooled slowly to 25 °C. Annealed double-stranded substrates were then separated from unannealed and excess single-stranded oligomers by nondenaturing 12% PAGE. Intact double-stranded DNA substrates were recovered using a Qiaex II gel extraction kit (Qiagen) and stored at 4 °C.

Helicase Substrates—PAGE-purified 44-mer and 19-mer oligonucleotides were purchased from the Midland Certified Reagent Company. The 19-bp duplex substrate with a 25-nucleotide 3′-tail (3′-overhang DNA substrate) was constructed by labeling 10 pmol of 19-mer oligonucleotide (5′-GTAAAACGACGGCCAGTGC-3′) at its 5′-end using T4 polynucleotide kinase and $[\gamma^{-32}P]ATP$. The labeled 19-mer was purified from unincorporated nucleotide by using a Microspin G-25 column from Amersham Biosciences. The 19-mer was mixed with 25 pmol of 44-mer oligonucleotide (5′-GCACTGGCCGTCGTTTTACGGTCGTGACTGGG-AAAACCCTGGCG-3′) in 50 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, heated at 100 °C for 5 min, then placed at 70 °C and allowed to cool slowly (~2 h) to 24 °C. The 19-bp duplex substrate with a 26-nucleotide 5′-fork and 25-nucleotide 3′-tail (forked DNA substrate) was
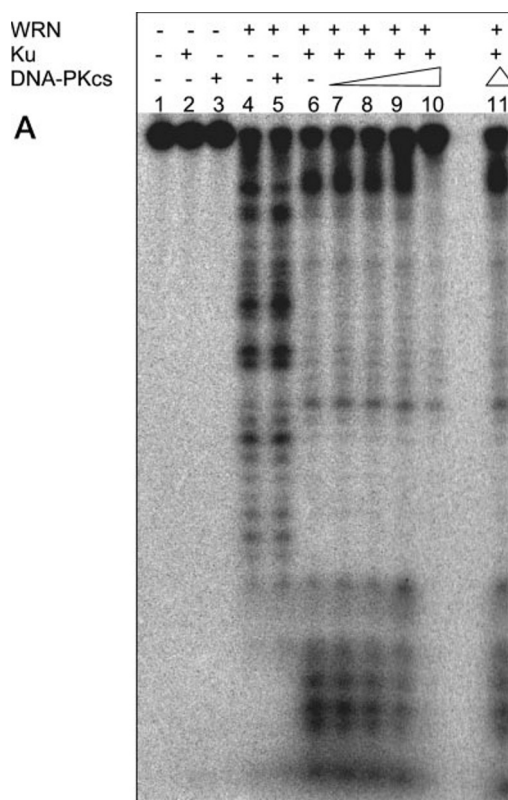
FIG. 1. **WRN exonuclease activity is inhibited by DNA-PKcs only in the presence of Ku.** *A*, 15 nM Werner protein was incubated with 3 fmol of exonuclease substrate in kinase buffer with 15 nM Ku and 0, 2.5, 5, 10, or 15 nM DNA-PKcs. The products were separated by 14% polyacrylamide gels under denaturing conditions and visualized by phosphorimaging. *B*, quantitative scan of the *lanes* in *A* using ImageQuant software (Molecular Dynamics). *H.I.*, heat-inactivated DNA-PKcs.

prepared using the same protocol except 10 pmol of 45-mer oligonucleotide (5′-TTTTTTTTTTTTTTTTTTTTTTTTCCAAGTAAAACGACGGCCA-GTGC-3′) was annealed to 25 pmol of the 44-mer oligonucleotide described above.

Exonuclease Assay

The assay to measure the 3′→5′ exonuclease activity of WRN was carried out in 10 μl at 37 °C with 40 mM Tris (pH 8.0), 5 mM MgCl$_2$, 1 mM dithiothreitol, 0.1 mg/ml BSA, 10 mM ATP (25). 3 fmol of exonuclease substrate was incubated with the indicated amount of Ku, DNA-PKcs, and WRN. The reactions were initiated by the addition of WRN and incubated at 37 °C for 60 min. Reactions were quenched by the addition of an equal volume of formamide loading buffer (80% formamide, 0.5× TBE, 0.1% bromphenol blue). The digestion products of these reactions were separated on denaturing 14% polyacrylamide gels and visualized using a PhosphorImager and quantitated using ImageQuant software (Molecular Dynamics).

Band Shift Assay

For DNA binding activity the solution conditions were the same as those used in the exonuclease assay. Approximately 3 fmol of ^{32}P-labeled exonuclease substrate was incubated on ice for 10 min with the indicated amount of Ku, DNA-PKcs, and WRN in buffer containing 40 mM Tris (pH 7.8), 5 mM MgCl$_2$, 1 mM dithiothreitol, 0.1 mg/ml BSA, and 10 mM ATP. 0.1% glutaraldehyde was added to the reaction, and incubation continued for 5 min more. 10 μl of DNA protein mixture was analyzed by 4% nondenaturing gel electrophoresis at 4 °C at 250 V with 1× Tris acetate buffer for 1.5 h. DNA was visualized using a PhosphorImager and analyzed using ImageQuant software.

In Vitro Phosphorylation

In vitro phosphorylation reactions were carried out either with the same exonuclease buffer and substrate or with kinase buffer (50 mM KCl, 1 mM dithiothreitol, 25 mM Hepes-KOH (pH 7.6), 5 mM MgCl$_2$, 50 μM ATP) with 100 ng of sonicated calf thymus DNA and 10 μCi of
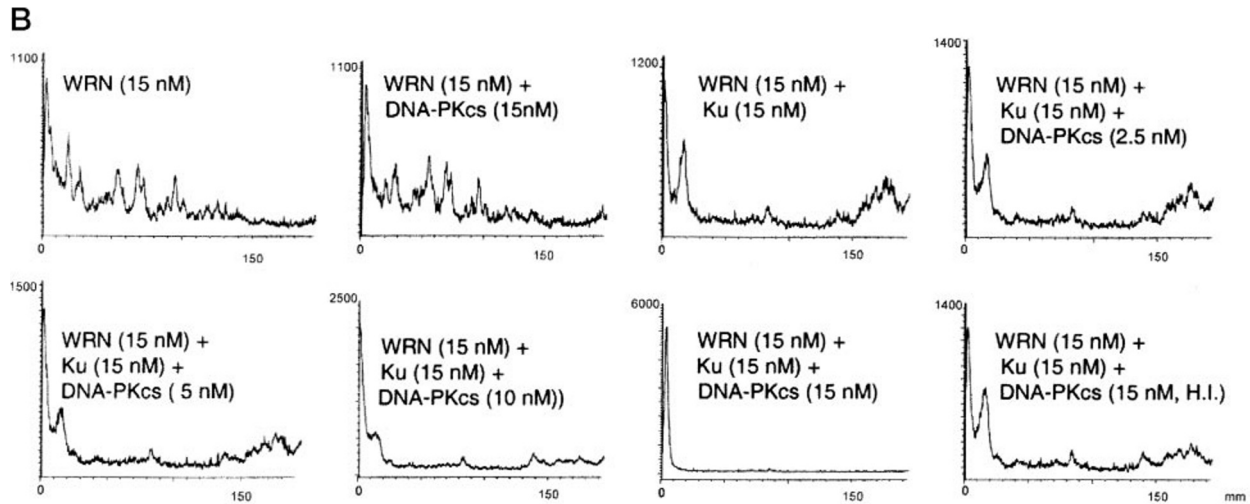
FIG. 1—*continued*

The reactions (10 μl) were initiated by the addition of DNA-PKcs and carried out at room temperature for 10 min. The reactions were stopped with the addition of 1 μl of 0.25 M EDTA. The resulting products were analyzed by electrophoresis on 4–12% acrylamide Tris-glycine gel (Invitrogen). The gel was washed and visualized as described above.

Phosphatase Reaction

2 nM WRN (final concentration) was incubated with the indicated amounts of PP1 in a 15-μl reaction (40 mM Hepes (pH 7.5), 7% glycerol, 50 mM KCl, 130 ng/μl BSA, 11 mM MgCl$_2$, and 3 mM ATP). The reaction was incubated for 10 min at 24 °C.

DNA Helicase Assay

Immediately after the phosphatase reaction, 10 fmol of the DNA helicase substrate (5 μl) was added to the reaction for a final volume of 20 μl (30 mM Hepes (pH 7.5), 5% glycerol, 40 mM KCl, 100 ng/μl BSA, 8 mM MgCl$_2$, and 2 mM ATP). The mixture was then incubated for 15 min at 37 °C. The reaction was terminated by the addition of 10 μl of 50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromphenol blue, and 0.1% xylene cyanol. The products of the helicase reaction were resolved by electrophoresis on 12% 1× TBE nondenaturing polyacrylamide gels. The radiolabeled DNA products were visualized using a PhosphorImager as described above. To determine the percentage of helicase substrate unwound, the following formula was used: % displacement = $100 \times P/(S + P)$, where P is the product volume, and S is the substrate volume. The values for P and S were corrected by subtracting background values in the no-enzyme and heat-denatured controls, respectively.

ATPase Assay

123 fmol of WRN was incubated with 1 microunit of PP1 in a phosphatase reaction buffer (40 mM Hepes (pH 7.5), 7% glycerol, 50 mM KCl, 130 ng/μl BSA, and 11 mM MgCl$_2$). The reaction was incubated for 10 min at 24 °C. In control reactions, phosphatase was omitted. Immediately after the phosphatase reaction, 100 ng of M13mp18 single-stranded DNA and 16 nmol of [^{3}H]ATP were added to the reaction for a final volume of 20 μl (30 mM Hepes (pH 7.5), 5% glycerol, 40 mM KCl, 100 ng/μl BSA, 8 mM MgCl$_2$). The reaction was then incubated for 15 min at 37 °C and subsequently quenched with the addition of 10 μl of stop solution (100 mM EDTA, 20 mM ATP, 20 mM ADP). Products of the ATPase reaction were resolved by thin layer chromatography and quantitated by scintillation counting.

Immunoprecipitation

For the *in vitro* reaction, purified WRN, Ku, and DNA-PKcs were incubated with cold exonuclease substrate and buffer at 37 °C for 15 min. The reaction mixture was then incubated with either goat polyclonal anti-WRN (Santa Cruz Biotechnology) or polyclonal anti DNA-PKcs (Santa Cruz Biotechnology) for 1 h at room temperature followed by a 1-h incubation with G protein. After washing the complex with 50 mM Hepes (pH 7.4) and 0.5% Triton X-100, the immunoprecipitated complex was separated in a 4–12% acrylamide Tris-glycine gel and transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with mouse monoclonal anti-Ku70/Ku80 (Santa

Cruz Biotechnology, 1:500), mouse monoclonal anti-WRN (PharMingen, 1:250), and mouse monoclonal anti-DNA-PKcs (Oncogene, 1:1000) antibody overnight at 4 °C followed by a 1-h incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000). The resulting signal was visualized with ECL plus a Western blotting detection system (Amersham Biosciences). Immunoprecipitation with total cell lysate was essentially the same as above; however, instead of purified proteins, cell lysate was used. Cell lysate was precleared with G protein-agarose prior to immunoprecipitation.

In Vivo Phosphorylation

HeLa cells were grown for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The human glioblastoma cell lines M059K (containing normal levels DNA-PKcs) and M059J (lacking expression of DNA-PKcs) were grown in Dulbecco's modified Eagle's medium and F-12 medium (Invitrogen) with 15% fetal bovine serum. After 24 h, ~2–5 × 10^6 cells were incubated with phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% dialyzed fetal bovine serum for 1 h to exhaust the pool of endogenous phosphate. The cells were then freshly supplemented with phosphate-free medium containing 100 μCi of ^{32}P$_i$ (DuPont) for 5 h. In some experiments, DNA-damaging agents bleomycin (1 or 5 μg/ml) or 4NQO (0.1 or 0.5 μg/ml) were added during the incubation period. In some experiments, cells were incubated with 25 μM wortmannin. The radiolabeling was terminated by washing the cells with ice-cold phosphate-buffered saline. The cells were then lysed with RIPA buffer (150 mM NaCl, 1% Triton, 0.1% SDS, 10 mM Tris (pH 8), 0.5% sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 units/ml DNase) supplemented with phosphatase inhibitors (1:1,000, Sigma). After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was precleared with G protein-agarose beads (Calbiochem) and incubated with 4 μg of polyclonal goat anti-WRN (from Santa Cruz Biotechnology) for 4 h. The immune complexes were collected by adding G protein-agarose beads and washed three times with RIPA buffer. Immunoprecipitated proteins were analyzed on 8–16% acrylamide Tris-glycine gels. The gels were washed and visualized by autoradiography.

RESULTS

DNA-PKcs Alone Does Not Affect WRN Exonuclease

We have shown previously that Ku can interact physically with WRN and functionally stimulate WRN 3′→5′ exonuclease activity (14). Because Ku and DNA-PKcs are subunits of DNA-PK, which is involved in the repair of DNA dsb, we explored the possibility that WRN exonuclease activity is modulated by DNA-PKcs. The exonuclease activity of WRN was assayed using a duplex DNA substrate containing a 5′-labeled, recessed 3′-end and one blunt end. DNA-PKcs alone has no effect on WRN exonuclease activity even at higher molar ratios (3.5:1) of DNA-PKcs to WRN (data not shown).

Inhibition of WRN Exonuclease Activity by DNA-PKcs in the Presence of Ku

Because Ku functionally modulates the activities of both WRN and DNA-PKcs, we assayed for the modulation of WRN exonuclease activity by DNA-PK. In Fig. 1*A*, 15 nM Ku and 15 nM WRN were incubated in the presence of different amounts of DNA-PKcs (2.5, 5, 10, and 15 nM) and exonuclease substrate in kinase buffer. 15 nM Ku alone or 15 nM DNA-PKcs alone has no effect on the substrate (*lanes 2* and *3*, respectively). WRN exonuclease activity was not altered by equimolar amounts of DNA-PKcs (*lane 5*). In *lane 6*, it was observed that Ku stimulated WRN exonuclease activity, as the degradation products were smaller in molecular size. We have reported this stimulation previously (14). To address the possibility that Ku heterodimer stimulates WRN exonuclease activity by enhancing the processivity of WRN exonuclease, we conducted unlabeled DNA competitor experiments. Unlabeled DNA substrate added in increasing amounts to ongoing WRN exonuclease reactions effectively quenched the WRN exonuclease reactions in the absence of Ku; however, the presence of Ku rendered the WRN exonuclease reaction resistant to quenching by a 10-fold molar excess of unlabeled DNA substrate (data not shown). These results suggest that the effect of Ku on WRN exonuclease is to render the enzyme more processive.

When DNA-PKcs was added to the reactions containing WRN and Ku, WRN exonuclease activity was inhibited markedly (Fig. 1*A*, *lanes 7–10*). At the highest concentration of DNA-PKcs used (corresponding to equimolar amounts of DNA-PKcs, Ku, and WRN) the WRN exonuclease activity was inhibited strongly (Fig. 1, *lane 10*). Similar results were also observed when kinase buffer was replaced by exonuclease buffer (data not shown). In Fig. 1*B*, we have scanned the lanes from the gel in Fig. 1*A*. It is evident from the patterns of digestion products shown by the scan analysis that DNA-PK inhibited WRN exonuclease activity. We also calculated the exonuclease activity based on the amount of undigested (full-length) substrate. It was $0.184 \pm 0.12\%$ fmol in the presence of 15 nM WRN. When 15 nM Ku was present this became $0.1 \pm 0.5\%$, and in the additional presence of 5, 10, and 15 nM DNA-PKcs, it became 0.55 ± 0.14, 0.8 ± 0.15, and $1.38 \pm 0.28\%$, respectively. The fraction of digested product ($\leq$20-mer) for the reaction containing WRN alone or WRN + DNA-PKcs was 42 ± 4 and $44 \pm 6\%$, respectively. In the presence of Ku and WRN this fraction became $64 \pm 6\%$. The addition of 5, 10, and 15 nM DNA-PKcs to the exonuclease reactions containing WRN and Ku yielded values of 57 ± 7, 48 ± 5, and $36 \pm 6\%$, respectively, for products $\leq$20-mer. These data, representing an average of three experiments, clearly demonstrate that DNA-PKcs and Ku together effectively inhibit the WRN exonuclease reaction. The addition of DNA-PKcs inhibits the ability of Ku alone to stimulate WRN exonuclease activity, instead reducing exonuclease activity to levels less than observed with WRN alone.

Incubation with Kinase Inhibitors Can Restore WRN Exonuclease Activity

DNA-bound Ku is required for maximal stimulation of the protein kinase activity of DNA-PKcs (26), and the protein kinase activity of DNA-PK is inhibited by 50–100 nM wortmannin or 50–100 μM LY294002 (27). The presence of wortmannin or LY294002 in the Ku/DNA-PKcs/WRN incubation reaction prevented the DNA-PK-dependent inhibition of WRN exonuclease activity (Fig. 2*A*, *lanes 6* and *7*). DNA-PKcs was preincubated with 100 nM wortmannin and 100 μM LY294002 to inhibit the DNA-PKcs kinase activity completely. In *lane 3*, it is shown again that DNA-PKcs itself did not affect the WRN exonuclease activity, whereas the inhibition of the exonuclease

in the presence of Ku and DNA-PKcs was observed (*lane 5*). Neither wortmannin nor LY294002 had any effect on WRN exonuclease activity or the Ku stimulation of the WRN exonuclease activity (data not shown). Thus, it appears that the protein kinase activity of DNA-PK is essential for the inactivation of WRN exonuclease activity.

C-terminal Ku80 Is Required for DNA-PK Inhibition of WRN Exonuclease Activity

To confirm that the protein kinase activity of DNA-PKcs was essential for inhibition of WRN exonuclease activity, we tested a form of DNA-PK which is defective in protein kinase activity. The C-terminal 12 amino acids of Ku80 have been shown to be essential for the interaction of DNA-PKcs with Ku (28). A mutant form of the Ku heterodimer with full-length Ku70 but missing the C-terminal 162 amino acids of Ku80 (Ku70/80ΔC) is fully able to bind DNA but does not support DNA-PK protein kinase activity.[2] When this mutant form of Ku was incubated with WRN and DNA-PKcs, there was no inhibition of WRN exonuclease activity (Fig. 2*B*). These data support the hypothesis that the active DNA-PK (DNA-PKcs + Ku) is required for abrogation of the exonuclease activity of WRN.

WRN Is Phosphorylated by DNA-PKcs Only in the Presence of Ku

We next tested whether WRN is a substrate of DNA-PK. Different combinations of WRN, Ku, and DNA-PKcs were incubated with sonicated calf thymus DNA as an effector and with [γ-^{32}P]ATP (Fig 3). Fig. 3 shows that WRN is phosphorylated only in the presence of both Ku and DNA-PKcs but not by either Ku or DNA-PKcs alone (*lanes 1* and *2*). The presence of wortmannin inhibited the phosphorylation of the four proteins (*lane 5*). DNA-PK phosphorylated Ku and autophosphorylated itself only when Ku was present (*lane 3*). Thus, we show that WRN can be phosphorylated by DNA-PK *in vitro* and that Ku is required for this phosphorylation. Phosphorylation of DNA-PKcs, Ku70, and Ku80 (Fig. 3, *lane 3*) represents autophosphorylation of DNA-PK (29). The WRN phosphorylation by DNA-PK was also confirmed under the reaction conditions for the exonuclease activity assay (data not shown), providing further evidence for the correlation between WRN phosphorylation and inhibition of its exonuclease activity.

Ku Mediates the Interaction between WRN and DNA-PKcs

To explore further the potential interactions among WRN, Ku, and DNA-PKcs, we conducted electrophoresis mobility shift assays, using the same buffers and substrate conditions as in the WRN exonuclease assay (Fig. 4). 5 nM Ku and 5 nM WRN were incubated with 1, 2.5, and 5 nM DNA-PKcs in the presence of exonuclease DNA substrate on ice for 10 min, and the resulting products were separated on 4% native polyacrylamide gels. Many studies have shown that Ku interacts with DNA substrates to form protein·DNA complexes in electrophoresis mobility shift assays. However, DNA-PKcs does not interact with DNA in this assay, and the interaction among DNA-PKcs, Ku, and DNA is weak in the absence of cross-linkers (30). Here, we used 0.1% glutaraldehyde to stabilize the complex. As expected, WRN or DNA-PKcs, separately or together, did not alter the mobility of the DNA probe (Fig. 4, *lanes 2*, *3*, and *5*, respectively), whereas the Ku·DNA substrate complex displayed a gel mobility shift (*lane 4*). When Ku was present with either DNA-PKcs or WRN, a supershift was observed (*lanes 6* and *7*, respectively). When all three proteins were present, a further mobility shift was observed. With increasing amounts
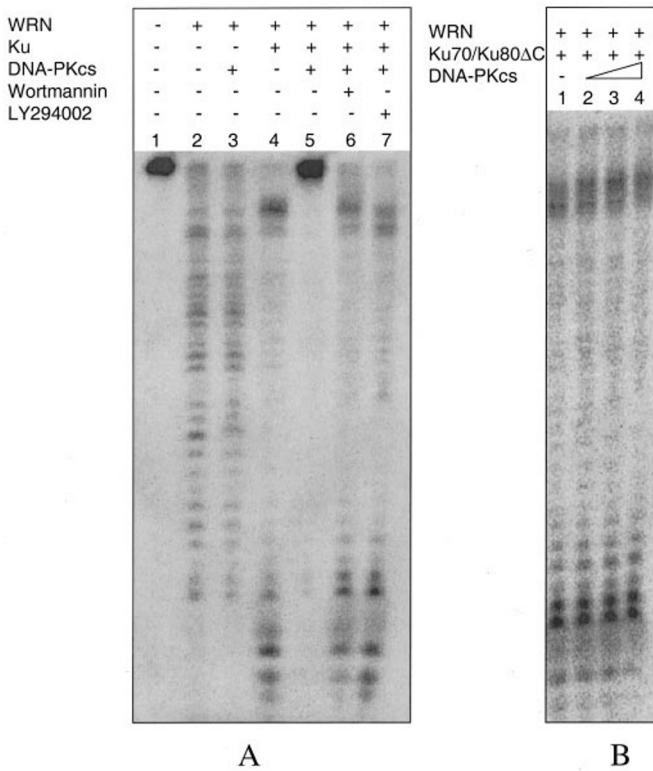

FIG. 2. *A*, the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 restore WRN exonuclease activity. Where indicated, 15 nM DNA-PKcs was preincubated for 10 min at room temperature with either 100 nM wortmannin or 100 μM LY294002 before addition to the reaction mixtures. Reaction mixtures contained 15 nM WRN protein, 15 nM Ku, 3 fmol of exonuclease substrate, and exonuclease buffer. The reactions were incubated for 1 h at 37 °C. The products were separated by 14% denaturing PAGE and visualized by phosphorimaging. *B*, Ku interaction with DNA-PKcs is necessary for inhibition of WRN exonuclease activity. 15 nM recombinant Ku70/Ku80ΔC in which the C-terminal 162 amino acids of Ku80 were deleted was incubated with 15 nM WRN and 5, 10, or 15 nM DNA-PKcs with exonuclease substrate (*lanes 2-4*, respectively). The products were separated on 14% polyacrylamide gels under denaturing electrophoresis, and the gels were visualized by phosphorimaging

of DNA-PKcs, the intensity of the supershifted bands was increased (*lanes 8*, *9*, and *10*). Interaction of the 156-kDa Ku heterodimer, 465-kDa DNA-PKcs, plus 167-kDa WRN formed a large protein·DNA complex that migrated a short distance into the gel. When treated with 1% SDS, the proteins dissociated, and the substrate was released (*lane 11*). The results suggest mutual bindings among WRN, Ku, and DNA-PKcs, and the possible interaction between DNA-PKcs and WRN would be mediated through Ku.

Physical Association between WRN and DNA-PKcs in Vivo and in Vitro

In vitro association of WRN with DNA-PK was investigated by mixing 5 nM purified WRN, Ku, and DNA-PKcs (final concentration of each) *in vitro* in the presence of exonuclease buffer and substrate, followed by immunoprecipitation with an antibody against WRN. The presence of WRN, Ku, and DNA-PKcs in the immunoprecipitated complex was determined by conventional Western blotting using the respective antibodies. As shown in Fig. 5*A*, WRN was physically associated with Ku (*lane 1*). Under our reaction conditions DNA-PKcs was not physically associated with WRN (*lane 2*). However, WRN was physically associated with DNA-PKcs in the presence of Ku (*lane 4*). Moreover, in the presence of DNase, the physical association between WRN and DNA-PK was still present and unchanged
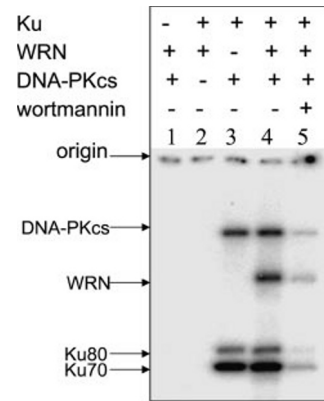

FIG. 3. **WRN phosphorylation requires Ku.** Ku, DNA-PKcs, and WRN (each at 15 nM) were incubated in combination with a kinase buffer, sonicated calf thymus DNA, and [γ-32P]ATP for 10 min at room temperature. The products were separated on 4–12% Tris-glycine acrylamide gels and visualized by phosphorimaging. *Lane 1*, WRN + DNA-PKcs. *Lane 2*, Ku + WRN. *Lane 3*, Ku + DNA-PKcs. *Lane 4*, WRN + Ku + DNA-PKcs. *Lane 5*, WRN + Ku + DNA-PKcs + 100 nM wortmannin.

(*lane 5*). Also, goat IgG did not immunoprecipitate any proteins from the purified mixture (*lane 8*). This experiment suggests that the interaction between WRN and DNA-PKcs is mediated by Ku.

To address whether this *in vitro* physical association also occurs *in vivo*, we immunoprecipitated this complex from a HeLa cell lysate. As seen in Fig. 5*B*, any of the three proteins can be immunoprecipitated by any of the three antibodies against WRN, Ku, or DNA-PKcs, indicating that they form a complex *in vivo*. As a control, we used WRN mutant skin fibroblasts cells, AG11395, where WRN antibody could not precipitate any protein (*lane 7*). However, in these mutant cells, Ku immunoprecipitated DNA-PKcs, and thus, as expected, the association between Ku and DNA-PKcs takes place in the absence of WRN (*lane 8*). Input was ~10% of the lysate used in each immunoprecipitation experiment. As a control we used goat IgG, which does not immunoprecipitate either of the proteins mentioned above from HeLa cell lysate (Fig. 5*B*, *lane 2*).

WRN Is Phosphorylated when Expressed in Insect Cells

Because DNA-PK phosphorylates substrates at Ser/Thr residues, we first determined whether recombinant WRN has such phosphorylation sites. Purified WRN recombinant protein was treated with PP1, which specifically removes phosphate from serine or threonine residues. As seen in Fig. 6*A*, the bands corresponding to WRN as detected by antibody to Ser/Thr phosphate disappeared with increasing amounts of PP1. Thus, recombinant WRN protein, when expressed in insect cells, contained phosphorylated residues at Ser/Thr.

Because it was possible that dephosphorylation of WRN could remove phosphate groups that were essential for recognition of WRN by DNA-PK, we first treated WRN (directly purified from insect cells) with PP1, then inhibited the protein phosphatase by adding sodium orthovanadate, and then determined whether DNA-PK could rephosphorylate the dephosphorylated WRN. As shown in Fig. 6*B*, pretreatment with PP1 (*lane 2*) did not affect the ability of DNA-PK to phosphorylate WRN at Ser/Thr residues (compare *lanes 3* and *4*). The antibody against Ser/Thr phosphate also detects bands corresponding to the phosphorylation of Ku and DNA-PKcs. The presence of an equal amount of WRN or Ku was verified (Fig. 6, *C* and *D*). We also show that PP1 can remove the phosphate, which is incorporated by DNA-PK by treating the WRN·DNA-PK complex with PP1 after the kinase reaction was stopped with wortmannin. As seen in Fig. 6*E*, the intensity of the incorpo-
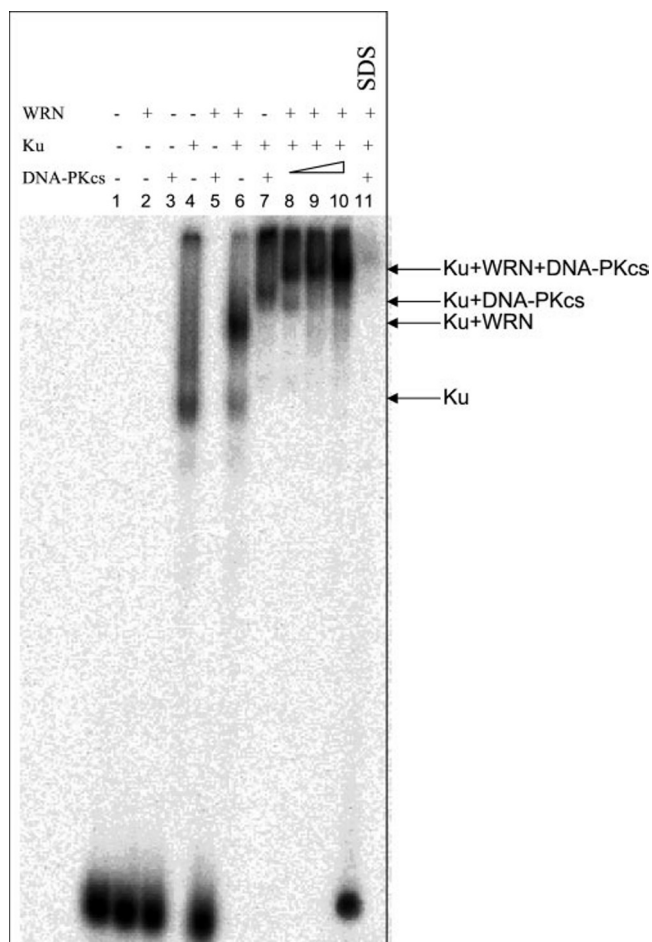
FIG. 4. **Ku mediates interaction between WRN and DNA-PKcs.** The DNA binding ability of 5 nM Ku, 5 nM WRN, and 1, 2.5, or 5 nM DNA-PKcs either alone or in combination was monitored by band shift assay. *Lane 1*, no enzyme. *Lane 2*, 5 nM WRN. *Lane 3*, 5 nM DNA-PKcs. *Lane 4*, 5 nM Ku. *Lane 5*, 5 nM WRN + 5 nM DNA-PKcs. *Lane 6*, 5 nM WRN + 5 nM Ku. *Lane 7*, 5 nM Ku + 5 nM DNA-PKcs. *Lanes 8–10*, 5 nM Ku + 5 nM WRN + 1, 2.5, and 5 nM DNA-PKcs. *Lane 11*, same as *lane 10*, but with 1% SDS.

FIG. 5. *A, in vitro* immunoprecipitation. 5 nM Ku, 5 nM WRN, and 5 nM DNA-PKcs were incubated in different combination with exonuclease substrate (unlabeled) and buffer. The protein mixture was immunoprecipitated with antibody against WRN or DNA-PKcs. *Lane 1*, WRN + Ku (immunoprecipitated with WRN antibody). *Lane 2*, WRN + DNA-PKcs (immunoprecipitated with WRN antibody). *Lane 3*, Ku + DNA-PKcs (immunoprecipitated with DNA-PKcs antibody. *Lane 4*, WRN + Ku + DNA-PKcs (immunoprecipitated with WRN antibody). *Lane 5*, WRN + Ku + DNA-PKcs + DNase (immunoprecipitated with WRN antibody). *Lane 6*, purified proteins. *Lane 7*, purified proteins. *Lane 8*, WRN + Ku + DNA-PKcs (immunoprecipitated with goat IgG). *B*, immunoprecipitation from cell lysate. HeLa cell lysate was immunoprecipitated with the antibody against WRN, Ku, or DNA-PKcs. *Lane 1*, HeLa cell lysate as input. *Lane 2*, HeLa cell lysate immunoprecipitated with goat IgG. *Lane 3*, HeLa cell lysate immunoprecipitated with WRN antibody. *Lane 4*, HeLa cell lysate immunoprecipitated with Ku antibody. *Lane 5*, HeLa cell lysate immunoprecipitated with DNA-PKcs antibody. *Lane 6*, WRN mutant AG11395 cell lysate as input. *Lane 7*, AG11395 cell lysate immunoprecipitated with WRN antibody. *Lane 8*, AG11395 cell lysate immunoprecipitated with Ku antibody. Input (either HeLa or AG11395, *lane 1* and *lane 6*, respectively) is ~10% of the lysate used in each immunoprecipitation experiment. *IB*, immunoblot.
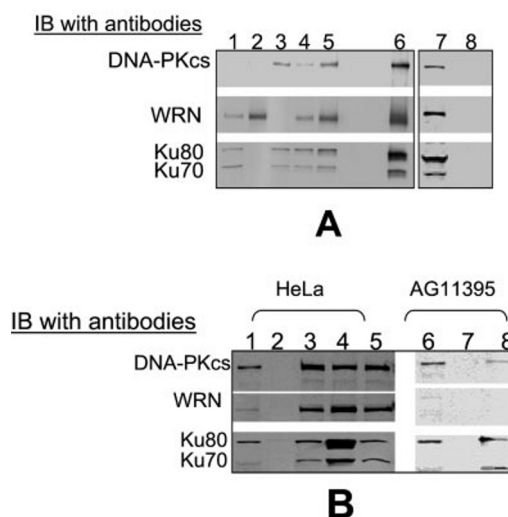
rated radioactive phosphate decreased after the PP1 treatment. This experiment clearly demonstrated that PP1 specifically removes phosphate, which was incorporated by DNA-PK, from WRN.

Given that DNA-PK phosphorylates WRN protein, we were next interested in whether this phosphorylation might affect any of the WRN catalytic activities. WRN has three characterized catalytic activities: an exonuclease function, a helicase function, and a DNA-dependent ATPase activity.

Dephosphorylation Enhances WRN Exonuclease Activity

We have shown that WRN is phosphorylated by DNA-PK *in vitro* and that the exonuclease activity of WRN is inhibited under conditions in which DNA-PK is active. PP1 has been shown to remove serine and threonine phosphate incorporated into DNA-PKcs and Ku by autophosphorylation (31). Protein phosphatase regulates DNA-PK activity. PP1 was shown to remove endogenous phosphate from baculovirus-expressed WRN (Fig. 6*A*) and from WRN that had been phosphorylated by DNA-PK *in vitro* (Fig. 6*E*). These data therefore suggested that PP1 could be used as a tool to characterize the effect of phosphorylation on the activities of WRN.

We next examined whether PP1 was able to modulate the exonuclease activity of WRN. As shown in Fig. 7*A*, it was observed that treatment of purified, recombinant WRN with

increasing amounts of PP1 enhanced the WRN exonuclease activity significantly (*lanes 3–6*). In the presence of WRN the percent of digested product ≤20-mer was 33 ± 5. When WRN was preincubated with PP1 the percent of digested product ≤20-mer increased with the amount of PP1 used (0.1 unit, 36 ± 7%; 0.25 unit, 39 ± 7%; 0.5 unit, 45 ± 4%; 1 unit, 56 ± 7%). Heat-inactivated PP1 did not affect WRN exonuclease (*lane 8*). Thus, dephosphorylation of baculovirus expressed WRN enhanced its exonuclease activity.

The stimulating effect of PP1 treatment on WRN exonuclease activity may be caused by the enhancement of enzyme processivity. To address this possibility, we tested the effect of adding unlabeled DNA substrate to an ongoing WRN exonuclease reaction. The exonuclease reaction was initiated by the addition of WRN that was either untreated or treated with 10 nM PP1 to 3 fmol of radiolabeled DNA substrate. After 2 min of incubation at 37 °C, specific amounts of unlabeled DNA substrate (3, 6, and 15 fmol) were added to the reaction, and the reactions were allowed to incubate for an additional 8 min. The products were resolved on denaturing polyacrylamide gels. As seen in Fig. 7*B*, the exonuclease activities of both WRN or PP1-treated WRN were inhibited by the presence of increasing amounts of unlabeled DNA substrate in a dose-dependent manner. Quantification of the scanned lanes yielded the percent of undigested substrate <43-mer for WRN with increasing amounts of unlabeled substrate DNA; at 0, 3, 6, and 15 fmol, the percents were 80 ± 3, 84 ± 1.8, 93 ± 2.6, and 95 ± 5, respectively, whereas the corresponding values for PP1-treated WRN were 53 ± 9, 63 + 2.4, 71 ± 8, and 86 ± 1.8%. These

results suggest that the processivities of WRN and PP1-treated WRN are similar. A similar effect of unlabeled DNA competitor on WRN exonuclease activity was observed when a higher concentration of WRN (30 nM) was used (data not shown). Thus, PP1 treatment of WRN did not enhance its processivity. Within the range of WRN phosphorylation status that we have been studying (before and after PP1 treatment), we found no change in DNA binding as observed by band shift assay (data not shown).

We have shown that DNA-PK could rephosphorylate WRN that had been PP1-treated, and we then asked whether rephosphorylation of WRN would affect the exonuclease function. As seen in Fig. 7*C* the exonuclease activity of WRN was increased after dephosphorylation (compare *lanes 2* and *3*). After rephosphorylation with DNA-PK (*lane 4*) the WRN exonuclease activity was strongly inhibited. In *lane 5*, we show that the Ku heterodimer stimulated the exonuclease activity of the dephosphorylated WRN. Thus, the presence of DNA-PKcs in the WRN-Ku reaction was responsible for the inhibition of WRN exonuclease activity. This experiment along with the experiment shown in Fig. 6*B* demonstrate that phosphorylation by DNA-PKcs can regulate WRN exonuclease activity.

Dephosphorylaton of WRN Enhances Its Helicase Activity on Duplex DNA Substrates

3′-Tailed Substrate—We next examined whether WRN Ser/Thr phosphorylation status affected its helicase activity. 1.5 nM WRN (final concentration) was incubated with 1 microunit of PP1 or PP1 storage buffer and analyzed for helicase activity. As shown on the gel (Fig. 8*A*, *lane 3*) WRN that was not treated with PP1 unwound 35% of the 3′-overhang helicase substrate. When WRN was pretreated with PP1 the unwinding increased to 58% (*lane 4*). This represents a 1.7-fold increase in WRN helicase activity on the 3′-overhang substrate for phosphatase-treated WRN compared with the untreated WRN protein. Importantly, PP1 did not destabilize the DNA substrate (Fig. 8*A*, *lane 2*), and heat-denatured PP1 was unable to enhance WRN helicase activity (data not shown). Also, 1 microunit of PP1 was unable to enhance the helicase activity of a WRN ATPase mutant protein (WRN K577M) that lacks helicase activity (32 and data not shown). Quantification of WRN helicase activity on the 3′-tailed duplex as a function of PP1 treatment is shown in Fig. 8*C*.

Forked Substrate—WRN was preincubated with PP1 or PP1 storage buffer and analyzed for helicase activity on a forked duplex substrate. In the absence of PP1, WRN catalyzed 8 and 12% unwinding of the forked duplex substrate at WRN concentrations of 100 and 380 pmol/liter, respectively (Fig. 8*B*, *lane 2* in each gel). 1 microunit of PP1 enhanced WRN helicase activity ~3.5-fold (28% substrate unwound) and 4.8-fold (57% substrate unwound) at WRN protein concentrations of 100 and 380 pmol/liter, respectively (Fig. 8*B*, *lane 3* in each gel). A quantitative representation of the data is shown in Fig. 8*D*.

The DNA-dependent ATPase activity of WRN was also tested after PP1 treatment. Although the other catalytic activities of WRN are enhanced significantly after PP1 treatment, there is no marked change in its ATPase activity (data not shown). Thus, the enhanced helicase activity of dephosphorylated WRN is not the result of a major change in its ATP-hydrolyzing activity.

Presence of Kinase Inhibitor or PP1 Treatment Does Not Dissociate the WRN·Ku·DNA-PKcs Complex

We next determined whether the presence of kinase inhibitor or PP1 treatment could affect the complex formation among WRN, Ku, and DNA-PKcs using electrophoresis mobility shift assays. The results are shown in Fig. 9*A*. *Lane 2* represents the
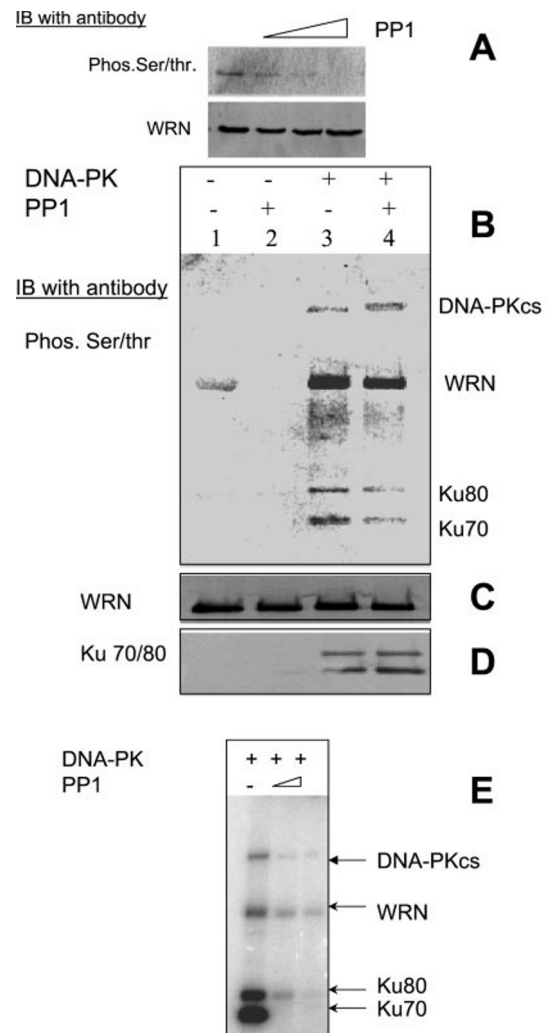

FIG. 6. *A*, WRN phosphorylation in insect cells. WRN protein is partially phosphorylated when expressed in insect cells. 10 nM purified WRN protein was treated with different amounts of PP1 (0.1, 0.5, and 1 unit) in phosphatase buffer for 10 min at room temperature and then separated on 4–12% acrylamide Tris-glycine gels. Conventional Western blotting was performed using antibody against Ser/Thr phosphate. The *upper panel* shows the bands as detected by antibody against Ser/Thr phosphate disappear gradually with increasing amounts of PP1. The same membrane was stripped and reprobed with WRN antibody to confirm that an equal amount of protein was present in the PP1 treatment, as seen in the *lower panel*. *B*, rephosphorylation of dephosphorylated WRN. 20 nM recombinant WRN was either mock-treated or treated with 0.5 unit of PP1 for 10 min at room temperature. The activity of PP1 was inhibited by adding 1 mM of sodium orthovanadate and followed by phosphorylation with DNA-PK. The reaction products were analyzed by conventional Western blotting using a Ser/Thr phosphate antibody. *Lane 1*, recombinant WRN mock-treated with PP1. *Lane 2*, recombinant WRN treated with PP1. *Lane 3*, PP1 mock-treated WRN followed by phosphorylation. *Lane 4*, PP1-treated WRN followed by phosphorylation. DNA-PK phosphorylates both PP1-treated and mock-treated WRN (*lanes 3* and *4*). The same membrane was stripped and reprobed with WRN antibody (*C*) and Ku 70/80 antibody (*D*). The mixture of 10 nM WRN, 10 nM Ku, and 10 nM DNA-PKcs was incubated with 10 μCi of [^{32}P-γ]ATP for 10 min at room temperature. The reaction was stopped by adding 100 nM wortmannin and followed by 0.25 unit and 0.5 unit of PP1 (*E*). The final reaction products were separated on a 4–12% Tris-glycine acrylamide gel, and bands were visualized by phosphorimaging. *IB*, immunoblot.

WRN·Ku·DNA-PKcs complex alone. With increasing concentrations of wortmannin (50, 100, 150 nM) the band pattern did not change (*lanes 3–5*). Also, the presence of 0.25, 0.5, and 1 unit of PP1 in the reaction mixture did not change the complex formation (*lanes 6–8*). Treatment with 1% SDS dissociates the com-
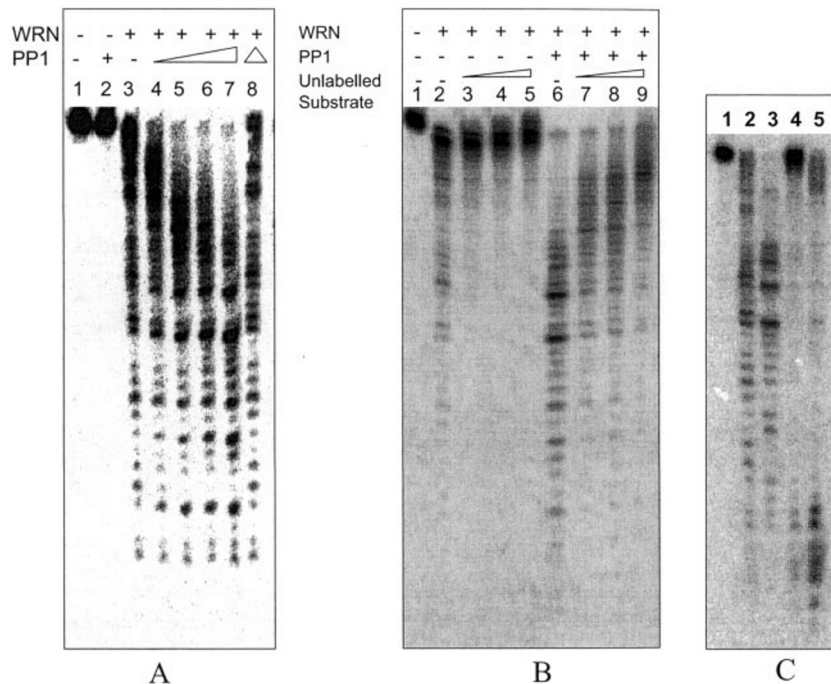
FIG. 7. *A*, dephosphorylation of WRN increases its exonuclease activity. 10 nM WRN protein was preincubated with PP1 or with PP1 storage buffer before using for exonuclease assay. The reaction conditions and substrate were the same as those in the exonuclease assay. The products were separated by 14% denaturing polyacrylamide gels and visualized by phosphorimaging. *Lane 1*, no enzyme control. *Lane 2*, exonuclease substrate with 1 unit of PP1. *Lanes 3–7*, WRN treated with 0, 0.1, 0.25, 0.5, and 1 unit of PP1, respectively, before the exonuclease reaction. *Lane 8*, effect of 1 unit of heat-inactivated PP1 on WRN exonuclease activity. *B*, dephosphorylation does not change the processivity of WRN. The exonuclease activity of PP1-treated or untreated WRN was assayed with increasing amounts of unlabeled DNA substrate. *Lane 1*, no enzyme. *Lane 2*, 10 nM WRN. *Lane 3*, WRN + 3 fmol of unlabeled DNA substrate. *Lane 4*, WRN + 6 fmol of unlabeled DNA substrate. *Lane 5*, WRN + 15 fmol of DNA substrate. *Lane 6*, 10 nM WRN treated with 0.5 unit of PP1. *Lane 7*, PP1-treated WRN + 3 fmol of unlabeled DNA substrate. *Lane 8*, PP1-treated WRN + 6 fmol of unlabeled DNA substrate. *Lane 9*, PP1-treated WRN + 15 fmol of unlabeled DNA substrate. *C*, phosphorylation of dephosphorylated WRN further inhibits WRN exonuclease activity. 10 nM WRN was incubated with either PP1 storage buffer or 0.5 unit of PP1 for 10 min at room temperature. PP1 was inactivated with the addition of 1 mM sodium orthovanadate. Later, the exonuclease substrate was added to the reaction with or without 10 nM Ku and 10 nM DNA-PKcs. *Lane 1*, no enzyme control. *Lane 2*, exonuclease activity of recombinant WRN. *Lane 3*, exonuclease activity of dephosphorylated WRN. *Lane 4*, presence of DNA-PK (Ku + DNA-PKcs) with dephosphorylated WRN. *Lane 5*, presence of Ku with dephosphorylated WRN. The reaction products were separated on a 14% denaturing polyacrylamide gel and visualized by phosphorimaging.

plex from the substrate (*lane 9*). We also found that the WRN·Ku·DNA-PKcs complex formed *in vitro* was unaffected by PP1 treatment (Fig. 9*B*). These results suggest that the physical association among WRN, Ku, and DNA-PKcs does not change in the presence of kinase inhibitor or after PP1 treatment.

WRN Is Phosphorylated in Vivo after DNA Damage

DNA-PK is required for the repair of DNA dsb induced by ionizing radiation, bleomycin, or other agents (20). Because WS cells are hypersensitive to 4NQO (9), an agent that produces oxidative DNA damage by the formation of radicals, which can ultimately cause DNA dsb, it is likely that WRN participates in this DNA damage response pathway. Indeed, the presence of both helicase and exonuclease activities within the WRN protein makes it an ideal candidate for processing of damage-induced DNA ends. We therefore tested whether WRN is phosphorylated *in vivo* in response to induced DNA damage.

HeLa cells were treated with bleomycin or 4NQO. As shown in Fig. 10, treatment with bleomycin induced phosphorylation of WRN in a dose-dependent manner (Fig. 10*A*). Treatment of the cells in culture with 4NQO also induced WRN phosphorylation (Fig. 10*B*). The *upper panels in* Fig. 10, *A* and *B*, show the silver staining of the gel, demonstrating that equal amounts of proteins were loaded. When the HeLa cells were preincubated with 25 μM wortmannin for 1 h before the addition of bleomycin, the amount of incorporated phosphate decreased significantly, suggesting that the activity of kinases belonging to the

phosphatidylinositol 3-kinase family were responsible for phosphorylation of WRN *in vivo* (Fig. 10*C*). To demonstrate that DNA-PKcs is a major kinase that phosphorylates WRN, we used human cell lines with (M059K) or without (M059J) DNA-PKcs (33). M059J are highly radiosensitive and fail to express DNA-PKcs because of a message instability (34). Bleomycin treatment of the cells induced phosphorylation of WRN only in M059K cells (Fig. 10*D*) and not in the DNA-PKcs-deficient cell line, M059J. Silver staining of the protein showed similar loading in the respective lanes. Thus, WRN phosphorylation occurs *in vivo* after DNA damage and is deficient in a cell line with loss of DNA-PKcs function.

DISCUSSION

We show that WRN is phosphorylated by DNA-PK *in vitro* and that DNA damage-induced phosphorylation of WRN is absent in a human cell line that lacks DNA-PKcs. We also show that the phosphorylation of WRN inhibits its exonuclease activity, whereas dephosphorylation of WRN enhances both its exonuclease and helicase activities. Thus, the Ser/Thr phosphorylation status of WRN plays a role in the regulation of its catalytic activities. Furthermore, a complex is formed between DNA-PK and WRN *in vivo* and *in vitro*, and this complex formation does not appear to be dependent upon phosphorylation status of these proteins. Thus, WRN protein is a target of DNA-PK phosphorylation, and the catalytic activities of WRN are regulated by phosphorylation. These observations suggest that WRN participates in a DNA-PK pathway of DNA metab-
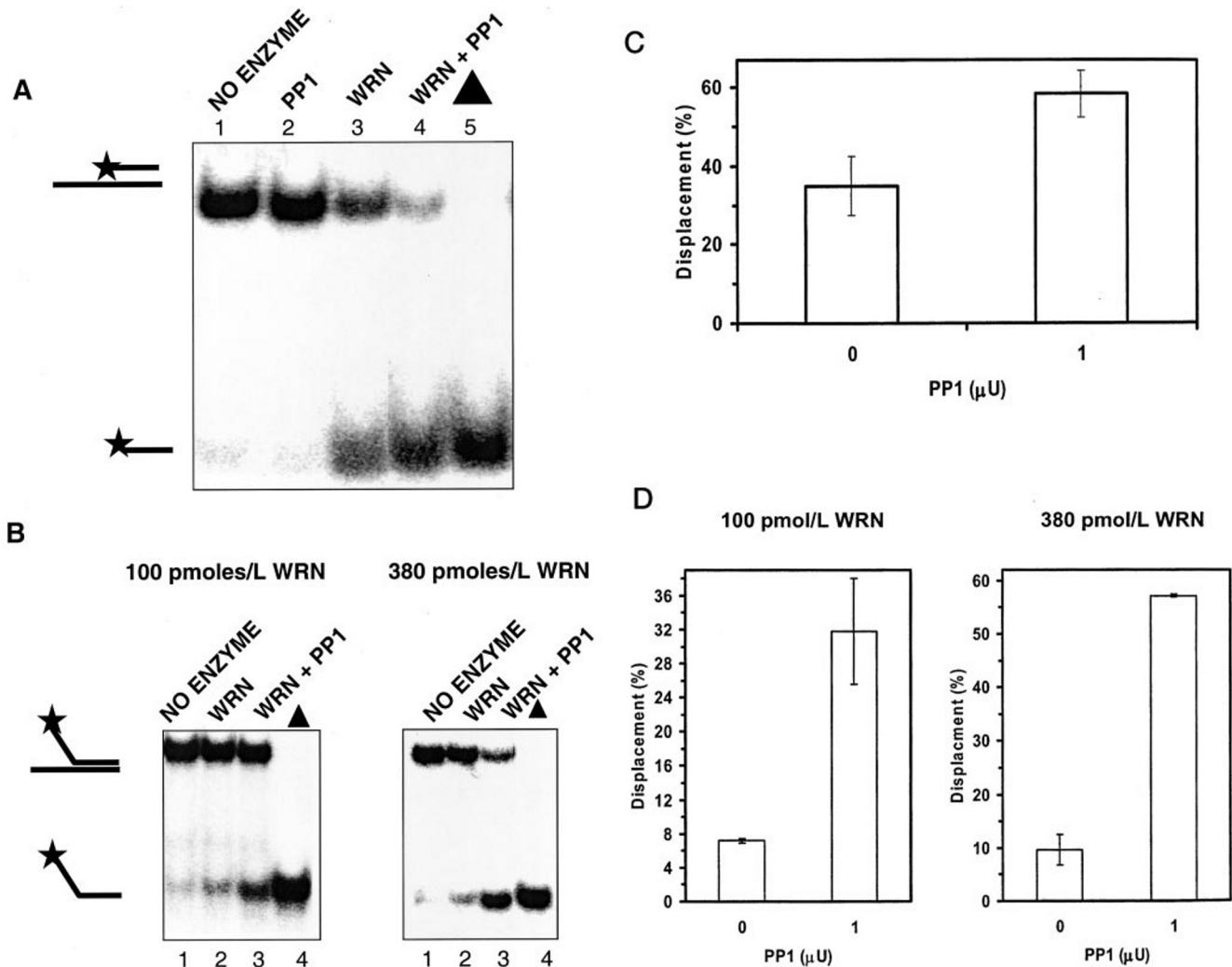
FIG. 8. *A*, dephosphorylation of WRN with PP1 enhances its helicase activity. 1.5 nM WRN (final concentration) was preincubated with 0 (*lane 3*) or 1 microunit (*lane 4*) of PP1 followed by incubation with a 3′-tailed duplex DNA substrate as described under "Experimental Procedures." *Lane 1*, substrate alone control. *Lane 2*, DNA substrate incubated with 1 microunit of PP1 but no WRN. *Lane 5*, heat-denatured DNA substrate control. Products were resolved by electrophoresis on a 12% nondenaturing polyacrylamide gel. *B*, WRN helicase activity on a forked DNA duplex is stimulated by dephosphorylation. 100 or 380 pmol/liter WRN protein (as indicated) was preincubated with PP1 storage buffer (*lane 2*) or 1 microunit of PP1 (*lane 3*) followed by incubation with a forked duplex DNA substrate as described under "Experimental Procedures." *Lane 1*, substrate alone control. *Lane 4*, heat-denatured helicase substrate control. Products were resolved by electrophoresis on a 12% nondenaturing polyacrylamide gel. *C* and *D*, quantitation of WRN helicase activity. *μU*, microunit. *C*, the percent substrate unwound by WRN as a function of PP1 is shown for the 3′-tail substrate. *D*, the percent substrate displaced by 100 or 380 pmol/liter WRN (as indicated) as a function of PP1 is shown for the forked duplex substrate. Data points represent the mean values of at least three independent experiments with standard deviations indicated by *error bars*.

olism. We find that the presence of kinase inhibitors or PP1 does not change the physical association between WRN and DNA-PK, and we conclude that physical association between DNA-PK and WRN is not critical for this inhibition of WRN exonuclease activity. Incubation with kinase inhibitors restores WRN exonuclease from DNA-PK-mediated inhibition.

DNA-PK is required for the repair of DNA dsb via the non-homologous end joining (NHEJ) pathway, and cells that lack DNA-PK are defective in DNA dsb repair. Although the precise mechanism of NHEJ has not been elucidated it is proposed that Ku binds to a DNA dsb and that DNA-PKcs is subsequently recruited to form an active protein kinase complex. Other proteins such as XRCC4 and DNA ligase IV are then recruited to ligate the DNA ends together. Although the physiological substrates of DNA-PK are not known, the protein kinase activity of human DNA-PKcs is required in DNA dsb rejoining (22), and ATP is required for NHEJ *in vitro* (35). Studies in cell-free extracts suggest that proteins in addition to DNA-PKcs, Ku, XRCC4 and DNA ligase IV are required for NHEJ. It is likely

that such additional proteins may play a role in processing DNA ends prior to ligation. Based on current models this processing is thought to involve both helicase and 3′→5′ exonuclease activity. The Mre11 component of the Mre11·Rad50·Nbs1 complex has 3′→5′ exonuclease activity and may function in NHEJ. It has been suggested that Ku might function as a helicase in NHEJ (26) based on previous observations that Ku can have helicase activity (36). However, in this laboratory, we have tested Ku activity on a variety of helicase substrates, and we have not found unwinding ability of the heterodimer on any substrate.[3] Because WRN has both helicase and 3′→5′ exonuclease activity and interacts with Ku and DNA-PK, WRN could potentially accomplish both of the putative processing steps of NHEJ. If WRN is essential in NHEJ, WS(−/−) cells would be expected to be more sensitive than normal cells to treatment

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[3] P. Karmakar, J. Piotrowski, R. M. Brosh, Jr., J. A. Sommers, S. P. Lees Miller, W.-H. Cheng, C. M. Snowden, D. A. Ramsden, and V. A. Bohr, unpublished results.

A

B
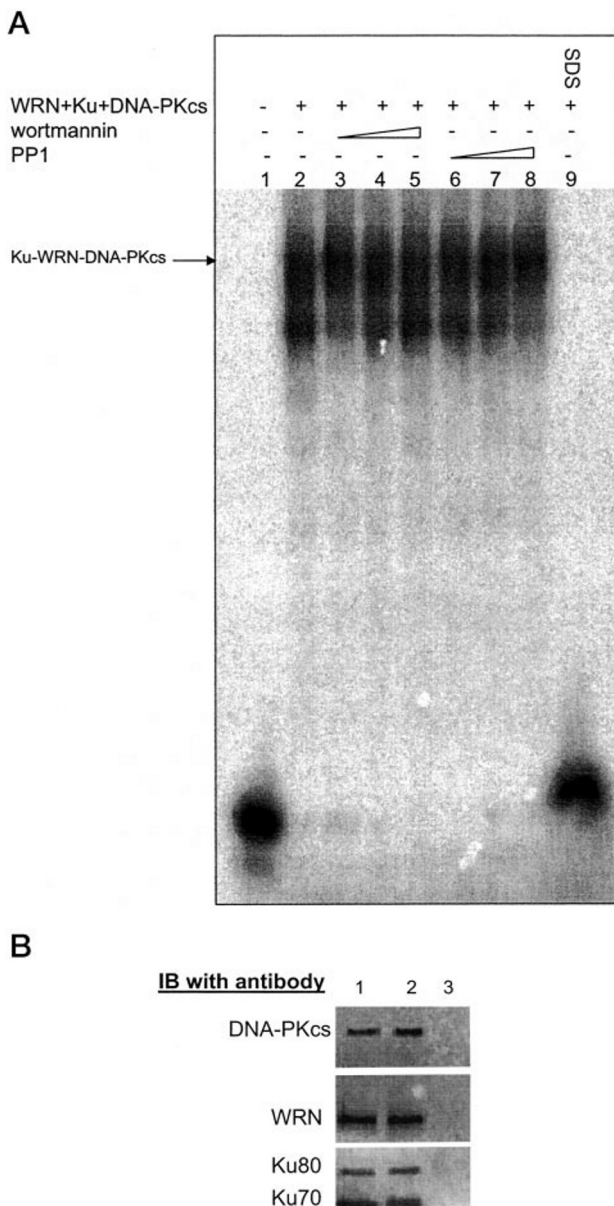
FIG. 9. *A*, the presence of kinase inhibitors or PP1 treatment does not dissociate WRN from DNA-PK. 5 nM DNA-PKcs, 5 nM WRN, and 5 nM Ku were incubated with wortmannin or PP1 at room temperature for 10 min with exonuclease substrate and buffer. The DNA-protein mixture was separated by a 4% nondenaturing polyacrylamide gel at 4 °C and visualized by phosphorimaging. 50, 100, and 200 nM wortmannin (*lanes 3*, *4*, and *5*, respectively) or at 0.1, 0.5, and 1 unit of PP1 (*lanes 6*, *7*, *8*, respectively) could not change the supershift over the free substrate by the WRN·Ku·DNA-PKcs complex. *Lane 9* represents same amount of WRN, Ku, and DNA-PKcs as in *lane 8*, except 1% SDS was added after the reaction. *B*, PP1 treatment does not change the physical association of WRN·Ku·DNA-PKcs. The *in vitro* formed complex WRN·Ku·DNA-PKcs (5 nM each) was treated with either PP1 storage buffer or 0.5 unit of PP1. The resulting products were immunoprecipitated with antibody against WRN and followed by immunoblot (*IB*) with antibody against WRN, Ku, or DNA-PKcs. *Lane 1*, WRN + Ku + DNA-PKcs (immunoprecipitated with WRN antibody). *Lane 2*, WRN + Ku + DNA-PKcs + PP1 (immunoprecipitated with WRN antibody). *Lane 3*, WRN + Ku + DNA-PKcs (no antibody).

with agents that introduce lesions that are removed via the NHEJ pathway. Some studies have shown a hypersensitivity of WS cells to γ-irradiation, but others have not (37). Recently, using a fluorescence *in situ* hybridization technique, it was demonstrated that x-rays could induce more DNA fragmentation in WS cells than in normal cells (38). WS cells are also hypersensitive to DNA cross-linking agents which ultimately can give rise

D
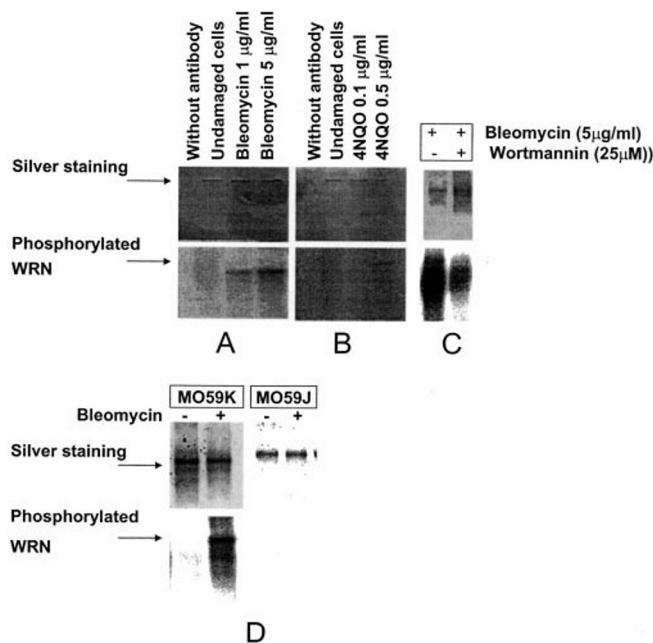
FIG. 10. **WRN is phosphorylated *in vivo* after DNA damage.** HeLa cells were incubated either with 1 or 5 μg/ml bleomycin (*A*) or 0.1 or 0.5 μg/ml 4NQO (*B*) for 5 h in the presence of 10 μCi/ml ^{32}P$_i$. The cells were then lysed and immunoprecipitated with antibody against WRN. Immunoprecipitated proteins were then resolved in a 8–16% acrylamide Tris-glycine gel. The gel was washed and visualized by phosphorimaging. Treatment of Hela cells with 25 μM wortmannin inhibits WRN phosphorylation after bleomycin treatment (*C*). The *upper panels* show the silver staining of the same gel to confirm that equal amounts of proteins were loaded. DNA-PKcs (+/+) M059K, and DNA-PKcs (−/−) M059J ganglioblastoma (*D*) cells were treated with 5 μg/ml bleomycin for 5 h in the presence of ^{32}P. The cells were lysed and immunoprecipitated with an antibody against WRN. The immunoprecipitated proteins were separated in a 8–16% acrylamide Tris-glycine gel and visualized by phosphorimaging. The *upper panels* show the silver staining of the gel confirming that equal amounts of protein were loaded.

to dsb (11). These studies suggest that WRN may have a role in repair of DNA damage which is repaired by NHEJ.

Recently Yannone *et al.* (39) reported that DNA-PKcs inhibited WRN exonuclease and helicase activity, and the additional presence of Ku alleviated this inhibition. The apparent discrepancy of those data with ours may be caused by the DNA substrates or purity of the proteins used in the two studies. Our results would suggest that contamination of the WRN or DNA-PKcs protein preparation with Ku results in the inhibition of WRN exonuclease activity mediated by DNA-PK phosphorylation of WRN. The concentration of DNA-PKcs used in the study by Yannone *et al.* (39) was 5–10-fold greater than that of WRN, raising the possibility that the inhibitory effect was nonspecific. We have shown that Ku-stimulated WRN exonuclease activity is inhibited markedly by the presence of DNA-PKcs and that the mutant Ku heterodimer (C-terminal 162-amino acid deletion of Ku80 subunit with full-length Ku70) that does not activate DNA-PK is also unable to inhibit WRN exonuclease activity in presence of DNA-PKcs (Fig. 2*B*). This particular Ku mutant retains its ability to stimulate WRN exonuclease activity (Fig. 2). Moreover, addition of kinase inhibitor to the WRN/Ku/DNA-PKcs reaction restored normal WRN exonuclease activity to the DNA substrate in the presence of Ku. This result suggests that catalytically active DNA-PK is required for inhibition of WRN exonuclease activity.

We show that WRN is phosphorylated *in vivo* after treatment of cells in culture with DNA-damaging agents. We have used two DNA-damaging agents, 4NQO and bleomycin. 4NQO is a carcinogen that introduces adducts, which are removed

primarily by nucleotide excision repair, and WS cells are hypersensitive to this agent (9). Bleomycin is an x-ray mimetic agent, and some sensitivity to it has been reported for WS cells (38). Bleomycin is known to cause various DNA modifications including DNA adducts, single strand breaks, and DNA dsb (40). We find that bleomycin induces more WRN phosphorylation *in vivo* than does 4NQO (Fig. 10). This supports our hypothesis that WRN plays a role in dsb repair. *In vivo* phosphorylation of WRN was ablated by the DNA-PK inhibitor wortmannin or in the cell line M059J, which lacks normal DNA-PKcs function. Because the M059J cells are also very low in ATM activity (41) we have used A-T cells (an ATM mutated cell line) to examine if ATM is responsible for the inhibition of WRN phosphorylation in the M059J cells. We find that the level of WRN phosphorylation appeared to be similar between the A-T cells and wild type fibroblast cells (data not shown). Yannone *et al.* (39) reported that WRN is phosphorylated by DNA-PK in undamaged Jurkat cells and human skin fibroblasts. As mentioned, we were not able to detect significant phosphorylation without exposing the cells to stress. This discrepancy may be caused by differences in cell type or procedure.

In addition to its role in recombination and DNA dsb repair, DNA-PK has also been implicated in several nuclear processes including transcription and DNA replication. Mutant Chinese hamster ovary cells lacking the Ku80 subunit or DNA-PKcs showed a reduced rate of transcription (42). Interaction of Ku with proteins involved directly in transcription initiation has also been observed (43, 44). Recently, it has been shown that Gcn5, a putative transcriptional adapter in human and yeast, is phosphorylated both *in vitro* and *in vivo* by DNA-PK and that this phosphorylation down-regulates the histone acetyltransferase activity of Gcn5 (45). We have shown previously that human WRN is directly associated with RNA polymerase II-mediated transcription and that WS cells have reduced transcription rates (46). Modulation of WRN function by phosphorylation could be part of the transcription process.

DNA-PKcs phosphorylates various proteins *in vitro*. These include p53, the large subunit C-terminal domain of RNA polymerase II, and replication protein A (20, 47). Phosphorylation by DNA-PK of the above proteins has only been shown *in vitro*, and we now demonstrate that WRN is a target for this phosphorylation *in vivo*. Physical and functional interactions between WRN and replication protein A (32) and WRN and p53 (48) have been demonstrated. It is possible that phosphorylation by DNA-PK regulates WRN and proteins that are associated with it. Further studies are required to investigate this, especially under conditions when cells have been exposed to DNA damage.

It is not uncommon that protein catalytic activities are regulated by phosphorylation. For example, the replication initiation protein MCM is phosphorylated by cdk2/cyclinA, which in turns inactivates its helicase activity (49). The intrinsic helicase function of NS1, the major nonstructural protein of the parvovirus minute virus in mice, is dependent on its phosphorylation (50). Phosphorylation of SV40 T antigen promotes double hexamer formation, a process to unwind the SV40 origin of replication (51). It appears that there is no uniform mode of regulation by phosphorylation, and some protein functions may be stimulated, whereas others decrease with phosphorylation. This regulation may depend upon the specific phosphorylation sites that are involved locally in different protein interactions, and there are three phosphorylation sites in WRN. We found the greatest increase in WRN helicase activity by dephosphorylation on the forked duplex substrate, a preferred substrate for WRN (52). Our finding that the phosphorylation state of WRN protein affects its helicase activity on that substrate

suggests that WRN engages this structure during specific events such as replication fork block. Fine tuned regulation of DNA unwinding at the fork by WRN phosphorylation is an attractive hypothesis, but further studies are required to address the functional importance of phosphorylation on WRN helicase activity *in vivo*.

Our finding that the level of phosphorylation of WRN greatly affects its catalytic activities has important implications for studies using this purified protein and perhaps for other helicase or exonuclease studies. It is possible that differences in observations regarding characteristics of the unwinding of different substrates by WRN could be explained by differences in the level of phosphorylation of the recombinant protein(s). This aspect should be considered in the biochemical studies of WRN and related proteins.

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