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The Werner Protein Acts as a Coactivator of Nuclear Factor κB (NF-κB) on HIV-1 and Interleukin-8 (IL-8) Promoters*

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Taketoshi Mizutani[‡1], **Aya Ishizaka**[‡], **and Yasuhiro Furuichi**[§]

From the [‡]*Laboratory of Virology, Institute of Microbial Chemistry, Microbial Chemistry Research Foundation (BIKAKEN), Tokyo, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021 and* [§]*GeneCare Research Institute Co., Ltd., 19-2 Kajiwara, Kamakura, Kanagawa 247-0063, Japan*

Background: Loss of WRN results in erroneous transcriptional regulation of certain genes; the underlying mechanistic details remain unclear.
Results: WRN is recruited to NF-κB-dependent HIV-1 LTR and IL-8 promoters and enhances their transactivation by activating RelA/p50.
Conclusion: WRN functions as a coactivator of RelA/p50.
Significance: WRN is directly involved in promoter-specific transcription.

The Werner syndrome helicase (WRN) plays a role in maintaining genomic stability. The lack of WRN results in Werner syndrome, a rare autosomal recessive genetic disorder, which causes premature aging accompanied by many complications such as rare forms of cancer and type 2 diabetes. However, the underlying mechanisms of these complications, arising due to the loss of WRN, are poorly understood. In this study, we demonstrated the function of WRN in transcriptional regulation of NF-κB targets. WRN physically interacts via its RecQ C-terminal (RQC) domain with the Rel homology domain of both the RelA (p65) and the p50 subunits of NF-κB. In the steady state, WRN is recruited to HIV-1 long terminal repeat (LTR), a typical NF-κB-responsive promoter, as well as the p50/p50 homodimer, in an NF-κB site-dependent manner. The amount of WRN on LTR increased along with the transactivating RelA/p50 heterodimer in response to TNF-α stimulation. Further, a knockdown of WRN reduced the transactivation of LTR in exogenous RelA/p50-introduced or TNF-α-stimulated cells. Additionally, knockdown of WRN reduced TNF-α stimulation-induced activation of the endogenous promoter of IL-8, an NF-κB-responsive gene, and WRN increased its association with the IL-8 promoter region together with RelA/p50 after TNF-α stimulation. In conjunction with studies that have shown NF-κB to be a key regulator of aging and inflammation, our results indicate a novel role of WRN in transcriptional regulation. Along with NF-κB, the loss of WRN is expected to result in incorrect regulation of downstream targets and leads to immune abnormalities and homeostatic disruption.

The Werner syndrome helicase protein (WRN)[2] partakes in a wide range of activities including DNA replication, telomere maintenance, double strand break repair, and activation of the p53 tumor suppressor (1, 2). The human *WRN* gene encodes a 1432-amino acid protein, which bears homology to the *Escherichia coli* RecQ DNA helicase (3). WRN also plays critical roles in DNA metabolism by facilitating cellular processes such as DNA recombination and repair in cooperation with other proteins, and the loss of WRN causes Werner syndrome (WS) (4). WS patients frequently die from several complications including lethal tumors. In WS patients, there is a higher incidence of non-epithelial tumors, such as soft tissue sarcoma, than that of epithelial carcinoma (5). In normal subjects, however, RecQ helicases including WRN seem to highly express in epithelial carcinoma and are required for their survival by maintaining genome stability, which leads to an idea that RecQ and WRN helicases are potential molecular targets for cancer therapy (6, 7).

A role of WRN in the regulation of RNAP II transcription has also been hypothesized (8, 9). This hypothesis was supported by the observation of a 40–60% reduction in transcription by RNAP II in WS lymphoblastic cells, which was rescued to a normal level by the addition of wild-type WRN protein into WS cell extracts (10). However, only scarce information is available about WRN target gene specificity and the mechanism by which WRN controls transcriptional activation of those genes, either directly or indirectly. Sharma *et al.* (11) reported a probable participation of WRN in retroviral transactivation and replication. In their study, WRN was reported to interact and cooperate with the Tat HIV-1 trans-activating protein to activate HIV-1 long terminal repeat (LTR) by recruiting histone acetyltransferase. Immortalized WRN-deficient WS fibroblast

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[1] To whom correspondence should be addressed. Tel.: 81-3-3441-4173; Fax: 81-3-3441-7589; E-mail: mizutanit@bikaken.or.jp.

[2] The abbreviations used are: WRN, Werner syndrome helicase protein; WS, Werner syndrome; LTR, long terminal repeat; RNAP II, RNA polymerase II; PCAF, P300/CBP-associated factor; P-TEFb, positive transcription elongation factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; RQC, RecQ C-terminal; RHD, Rel homology domain; TAD, trans-activating domain; SASP, senescence-associated secretory phenotype; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

cells were found to exhibit comparable defects in recruiting PCAF and P-TEFb to HIV-1 LTR, and because of this, the WRN helicase was concluded to participate in the recruitment of PCAF/P-TEFb-containing transcription complexes to HIV-1 LTR via the Tat protein. Interestingly, the exogenous WRN expression was shown to increase viral transactivation without Tat. This observation suggested the possibility of the existence of another unidentified mechanism for the participation of WRN in transcriptional regulation.

Transcription of the HIV-1 provirus is characterized by early Tat-independent and late Tat-dependent phases. HIV-1 transcription depends on the interaction of host transcription factors with cis-regulatory DNA elements in the viral 5′ LTR and assembly of the transcription apparatus, which includes NF-κB, SP1, and RNAP II, in the early Tat-independent phase (12). NF-κB is composed of homo- or heterodimeric complexes that include members of the NF-κB protein family such as RelA (p65), RelB, c-Rel, p50, and p52 in humans (13), which play a central role in the transactivation of the HIV-1 LTR. In the steady state, the RelA/p50 heterodimer is bound by a specific inhibitor (IκBα) and remains inactive in the cytoplasm (14). The p50/p50 homodimer and the histone deacetylase complex-1 (HDAC1) predominantly bind to the NF-κB-responsive sites on HIV-1 LTR as well as certain endogenous NF-κB-responsive genes (15, 16). This complex negatively regulates basal transcription via histone modification, and several studies have indicated the crucial role of histone modification in the transcription of innate immunity and inflammation responses (17, 18). Upon exogenous stimulation, p50/p50-HDAC1 complex is replaced by transactivating RelA/p50 heterodimer that is released from IκB, which is rapidly transported into the nucleus (15, 16).

There has been increasing evidence concerning an active cross-talk between senescent and neighboring cells via secretory factors such as growth factors, extracellular proteases, cytokines, and chemokines (19). These phenomena are collectively known as senescence-associated secretory phenotype (SASP) (20, 21). IL-6 and IL-8, which are transcriptionally regulated by NF-κB, are major SASP proteins (22). These are considered to play a crucial role in inflammation and aging and to have a pro-oncogenic effect on surrounding pre-malignant cells (23–26).

In the present study, we demonstrate the interaction of WRN with p50 and its subsequent recruitment to the κB sites of the HIV-1 promoter in the steady state. Furthermore, a physical interaction of WRN with RelA resulting in the enhancement of viral transactivation, mediated by RelA/p50 activation after TNF-α stimulation, is demonstrated. We also observed the recruitment of WRN to the endogenous IL-8 promoter and its NF-κB-dependent activation. In summary, our results provide convincing evidence for the direct involvement of WRN in promoter-specific transcription.

Experimental Procedures

Cell Culture and Plasmid Construction—293FT cells and HeLaS3 cells in DMEM containing 10% fetal calf serum (Life Technologies) were maintained at 37 °C and 5% CO_2. 293FT-LTR-Luc-5 has been previously described in detail (27). The complete sequence encoding the N-terminal HA tag fused to WRN cDNA was inserted into the expression vector pcDNA3 (Life Technologies). The pCold-GST-His_6 vector was generated by inserting GST fragment (0.3 kb at DraIII-BamHI sites) in pCold-I vector (TAKARA Bio Ltd., Shiga, Japan), and then a His_6 fragment was inserted between the XhoI and NotI restriction sites. The pCold-GST-His_6-WRN-mt1, -mt2, -mt3, and -mt4 vectors were generated as follows. Each partial fragment of WRN was amplified by PCR, and these fragments were introduced at BamHI and XhoI sites in the pCold-GST-His_6 vector to generate GST and His_6 fusion proteins. Mutated NF-κB was generated using site-directed mutagenesis as described in a previous study (28). Short hairpin RNAs were derived from mouse U6 promoter. The target sequences of short hairpin RNA used in this study are as follows: shWRN, 5′-GGGCACCAAAGAGCATTGTTA-3′, and shLacZ, 5′-GCAGTTATCTGGAAGATCAGG-3′,

Transfection and Luciferase Analysis—Cells were transfected with plasmid DNA using FuGENE HD (Roche Applied Science, Basel, Switzerland) and Lipofectamine™ 2000 (Life Technologies). At 48 h after transfection, whole cell lysates were used for Western blotting and luciferase assays. Luciferase assay was performed by a GloMax™ 96 microplate luminometer (Promega Corp., Madison, WI).

Antibodies—The following antibodies were used in this study: anti-WRN (4H12), anti-p105/50 (ab7971) (Abcam, Cambridge), anti-RelA (C-20) and anti-HDAC1 (SC7872) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (013-24553) (Wako Ltd., Osaka, Japan), and LARP7 (A303-723A) (Bethyl Laboratories Inc.). In Western blotting experiments, band images were analyzed using the LAS 4000 UV mini system (Life Technologies).

TNF-α Stimulation—293FT and 293FT-derived cells were stimulated by TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN), and cell lysates were then collected at each time point after stimulation.

ChIP Assay—ChIP assays were performed as described previously (27). Briefly, lysed cells were treated with 1% formaldehyde to cross-link protein DNA complexes, and then lysates were sonicated on ice using ELESTAIN035SD (ELECON Science Corp., Chiba, Japan) to shear the DNA into small fragments with an average length less than 0.5 kb. Lysates were incubated overnight on a rotating platform at 4 °C with respective antibodies (5 μg each), which were previously bound to Dynabeads® Protein G (Life Technologies). After washing, DNA was purified, and quantitative PCR was performed using the CFX96 system (Bio-Rad) with Premix Ex Taq (probe quantitative PCR and SYBR quantitative PCR; Takara Bio Ltd.). Threshold values (Ct) were calculated, and all reactions were run in triplicate. The specific primer pairs and probes used in this study are as follows: NF-κB (HIV-1 promoter), forward, 5′-CTAGCATTTCATCACGTGGC-3′, reverse, 5′-CAGCGGAAAGTCCCTTGTAG-3′, and probe, 5′-FAM-AGCTGCATCCGGAGTACTTC-TAMRA-3′; and IL-8 promoter (NF-κB region), forward, 5′-AGGAAGTGTGATGACTCAGG-3′, and reverse, 5′-CACCCTACTAGAGAACTTATGCAC-3′.

RNA and Genomic DNA Preparation—Total RNA was prepared from cells using the Isogen II isolation kit (NIPPON

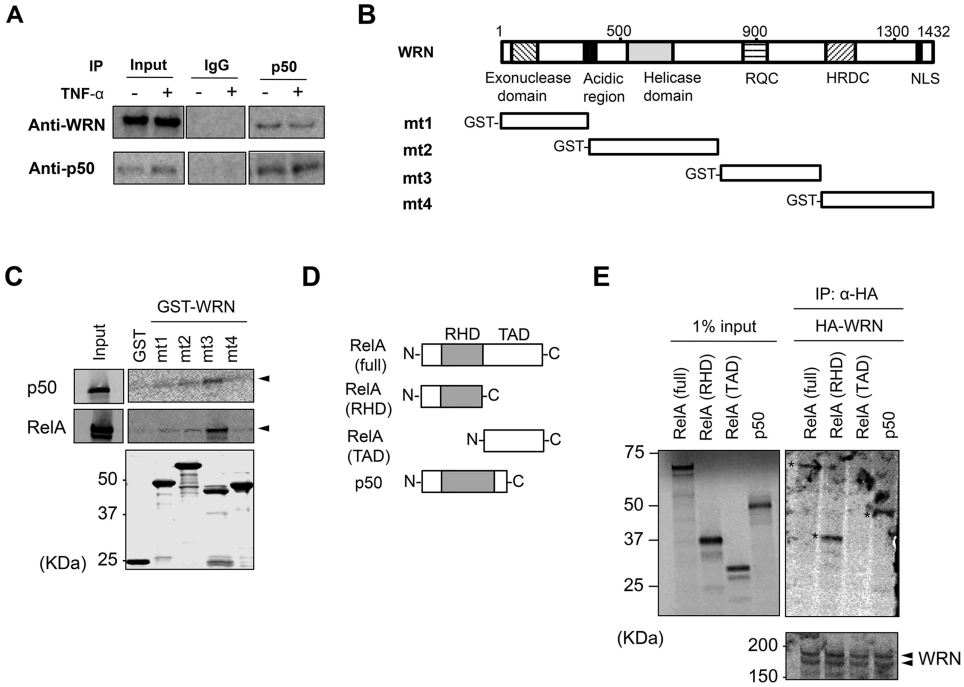

FIGURE 1. **WRN interacts with both RelA and p50 through its RQC domain.** *A*, immunoprecipitation (*IP*) assay for the WRN and p50 interaction. HeLaS3 cells were treated with TNF-α stimulation (10 ng/ml, 0 or 1 h), and nuclear fractions of HeLaS3 cell lysates were harvested for immunoprecipitation with the anti-p50 antibody. Immunoprecipitates were analyzed by Western blotting using anti-p50 or WRN antibodies. *B*, a schematic representation of the WRN protein and GST-fused deletion constructs of WRN (WRN-mt1, -2, -3, and -4). *HRDC*, helicase and RNase D C terminus; *NLS*, nuclear localization signal. *C*, GST pulldown assay. Recombinant GST-WRN derivative proteins were purified from *E. coli* lysates and Coomassie Blue-stained after SDS-PAGE (*lower panel*). Equal amounts of recombinant GST-WRN derivative proteins were incubated with ^{35}S-labeled RelA and p50 translated *in vitro* using a reticulocyte *in vitro* translation system. Following incubation and washing, GST beads were eluted with SDS sample buffer, and eluates were subjected to SDS-PAGE and autoradiography. The input was 2% of the labeled RelA and p50 in each reaction (*upper* and *middle panels*). *D*, a schematic representation of RelA, RelA derivatives, and p50. *E*, immunoprecipitation assay. ^{35}S-labeled RelA, RelA derivatives, p50, and HA-tagged WRN were translated *in vitro*. HA-tagged WRN and each recombinant protein were incubated together and then subjected to immunoprecipitation using HA antibody-conjugated beads. After precipitation and washing, immunoprecipitation products were eluted with SDS sample buffer, and eluates were analyzed with SDS-PAGE and autoradiography. * indicates the precipitated bands.

GENE, Tokyo, Japan). These isolated RNA were treated with Turbo DNase (Ambion, Austin) in accordance with the manufacturer's instructions. CDNA was synthesized from total RNA by first-strand cDNA synthesis using the PrimeScript RT master mix (Takara Bio Ltd.). Genomic DNA was collected using MagExtractor -Genome (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. Quantification of proviral DNA copy was normalized by endogenous GAPDH genomic DNA copy.

Quantitative PCR—Quantitative PCR was performed with a CFX96 System (Bio-Rad) using Premix Ex Taq (probe quantitative PCR) or SYBR Premix Ex Taq (Takara Bio Ltd.). Threshold values (Ct) were calculated, and all reactions were run in triplicate. The specific primer pairs and probes used in this study are as follows: HIV-1 elongated transcripts, forward, 5′-CAATAAAGCTTGCCTTGAGTG-3′, reverse, 5′-GCCACTGCTAGAGATTTTCC-3′, and probe, 5′-FAM-CTCTGGTAACTAGAGATCCC-TAMRA-3′; *IL-8* transcripts, forward, 5′-CTCTCTTGGCAGCCTTCCT-3′, and reverse, 5′-GGGTGGAAAGGTTTGGAGTA-3′; and GAPDH genomic DNA (promoter region), forward, 5′-CCTCCAAGGAGTAAGACCCC3′, and reverse, 5′-TGTGAGGAGGGGAGATTCAG-3′.

In Vitro Binding Experiments—GST pulldown assays were performed as described previously (27). [^{35}S]Methionine-labeled RelA or p50 constructs were translated using a reticulocyte lysate system *in vitro* (Promega) and incubated with GST alone or GST-His-tagged WRN derivative deletion constructs. These interactions were analyzed by SDS-PAGE followed by autoradiography.

Immunoprecipitation Assay—Immunoprecipitation assays were performed as described previously (27). Briefly, cells were lysed with TNE buffer (10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and protease inhibitors, and cell lysates were incubated overnight on a rotating platform at 4 °C with respective antibodies (5 μg each), which were previously bound to Dynabeads® Protein G (Life Technologies). After washing, precipitates were purified, subjected to SDS-PAGE, and analyzed by Western blotting or autoradiography.

Statistical Analyses—We performed a Student's *t* test. All statistical tests were two-sided. We considered *p* values less than 0.05 to be statistically significant.

Results

WRN Interacts with RelA and p50 in Vitro via the RQC Region—To elucidate the interaction of endogenous WRN and p50 (NF-κB1), we performed immunoprecipitation using nuclear extracts obtained from HeLaS3 cells (Fig. 1*A*). Following immunoprecipitation, endogenous WRN was detected by Western blotting with an antibody directed against p50, both with and without TNF-α stimulation. This suggested a consistent interaction of WRN with p50 through transactivation *in vivo*. Next, we evaluated the direct interaction of WRN with p50 and investigated the specific region of WRN necessary for mediating this interaction. WRN was divided into four regions,

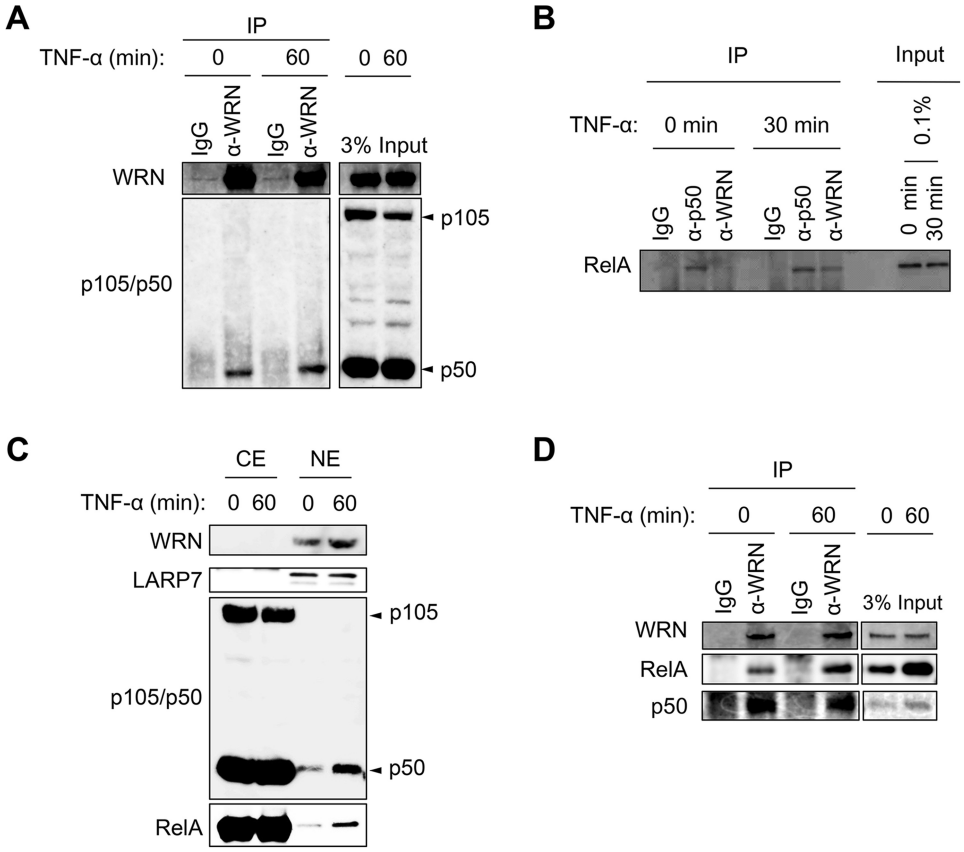

FIGURE 2. **WRN interacts with both RelA and p50 in nucleus in a TNF-α-dependent manner.** *A*, immunoprecipitation (*IP*) assay for the interaction between WRN and p105/p50 using whole HeLaS3 cell lysate with or without TNF-α (10 ng/ml, 0 or 60 min) treatment. *B*, immunoprecipitation assay for WRN and RelA interaction *in vivo*. HeLaS3 cells were treated with TNF-α (10 ng/ml, 0 or 30 min), and HeLaS3 whole cell lysates were harvested for immunoprecipitation with anti-p50 and anti-WRN antibodies. Immunoprecipitates were analyzed by Western blotting using the RelA antibody. *C*, Western blotting analysis using nuclear (*NE*) or cytoplasmic (*CE*) extracts in HeLaS3 cells with or without TNF-α (10 ng/ml, 0 or 60 min) treatment. *D*, immunoprecipitation assay for the interaction between WRN and either RelA or p50 using nuclear cell lysate in HeLaS3 cells with or without TNF-α (10 ng/ml, 0 or 60 min) treatment. Immunoprecipitates were analyzed by Western blotting using the indicated antibodies.

each of which was generated as GST fusion protein (Fig. 1*B*) and immobilized on glutathione-Sepharose beads (Fig. 1*C*, *bottom panel*). ^{35}S-labeled p50 translated *in vitro* bound primarily to GST-WRN-mt3, the region containing the RecQ C-terminal (RQC) domain (Fig. 1*C*, *upper panel*). Interestingly, the NF-κB family protein RelA, a heterodimeric partner of p50, also associated with GST-WRN-mt3 (Fig. 1*C*, *middle panel*). This indicated that WRN interacted with both p50 and RelA via its RQC domain *in vitro*. Given that both RelA and p50 possess a Rel homology domain (RHD), but only RelA possesses a trans-activating domain (TAD), the RHD domain was expected to mediate the interactions with GST-WRN-mt3. We generated [^{35}S]methionine-labeled recombinant proteins of RelA deletion mutants that contained either RHD or TAD domain as well as full-length RelA and p50 (Fig. 1*D*). Immunoprecipitation experiments using α-HA antibody were performed to analyze the interaction between the recombinant RelA deletion mutants and [^{35}S]methionine-labeled HA-tagged WRN. It was observed that WRN interacted with the RHD-containing proteins (*i.e.* full-length RelA, p50, and the RHD domain of RelA) (Fig. 1*E*). Taken together, these findings indicate that the RQC region of WRN interacts with the RHD domain of both p50 and RelA *in vitro*.

WRN Interacts with RelA and p50 in Nucleus in a TNF-α-dependent Manner—We next asked whether WRN interacts with NF-κB in cell lysates. We performed a co-immunoprecipitation assay using the whole cell lysate from HeLaS3 cells with or without TNF-α stimulation. As shown in Fig. 2*A*, the p50 protein was consistently co-immunoprecipitated by an antibody against WRN. However, we observed no association between WRN and p105, the precursor protein of p50, suggesting that WRN distinguishes p50 from p105 *in vivo*. In contrast, we found the stimulation-dependent interaction of WRN with RelA using the whole cell lysates (Fig. 2*B*). Given that the p50 homodimer is present in the nucleus in unstimulated cells (15, 16) and that the RelA/p50 heterodimer translocates to the nucleus in a stimulation-dependent manner (14), the association between WRN and NF-κB is likely to occur in the nucleus. To examine this possibility, we further analyzed the interactions using the nuclear extracts. The accuracy of the fractionation was confirmed by LARP7 (La-related protein 7, nuclear localization (29)) and NF-κB p105 (cytoplasmic localization (14)) (Fig. 2*C*). We observed that WRN constitutively localizes in the nucleus, whereas RelA and p50 was confirmed to be imported into the nuclear compartment from the cytosol after TNF-α stimulation (Fig. 2*C*). We then performed a co-immu-

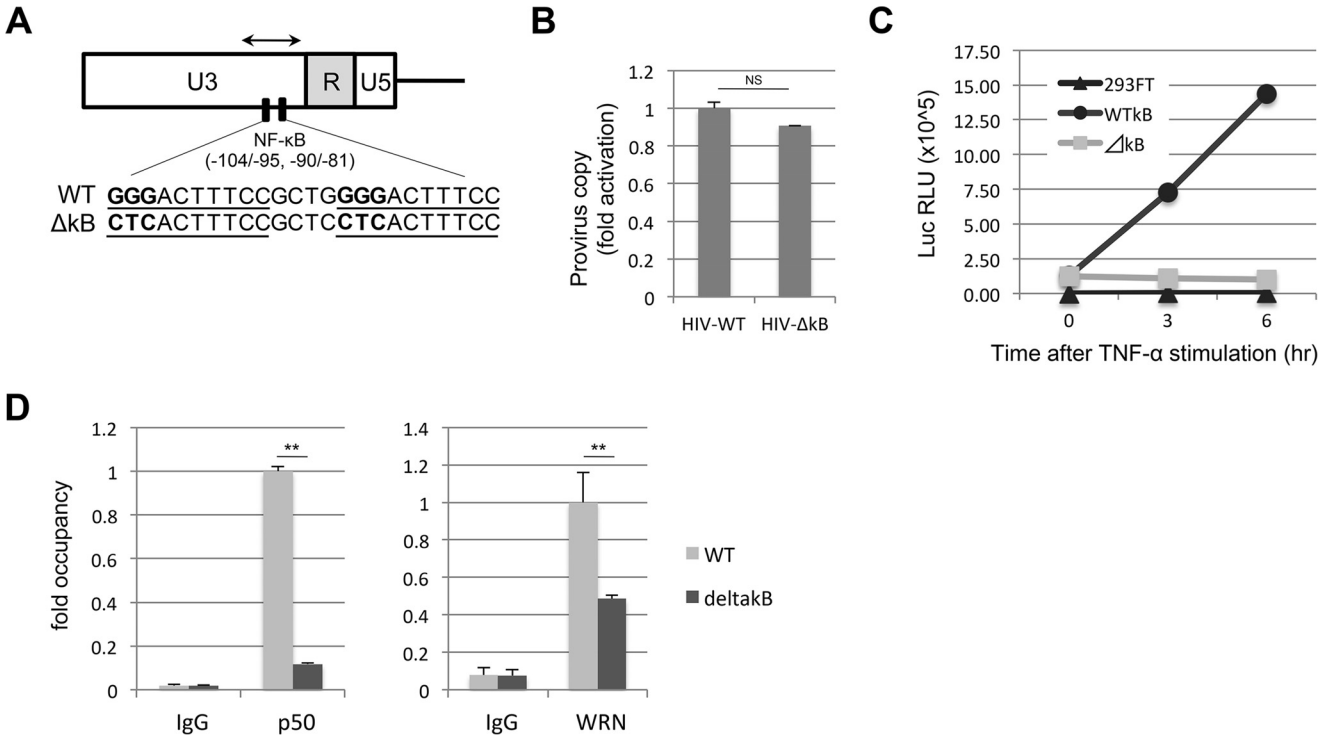

FIGURE 3. **WRN is recruited to the HIV-1 promoter via NF-κB sites.** *A*, a schematic representation of wild-type (wtκB) and mutated (Δκ B) κB sequences in the HIV-1 promoter. NF-κB consensus sequence is *underlined* in HIV-1 LTR. *B*, comparing proviral copies between wtκB- and Δκ B HIV-1 reporter vectors by quantitative PCR. *C*, comparing luciferase activity between wtκB- and Δκ B HIV-1 reporter vectors upon TNF-α stimulation. *D*, ChIP assay of relative WRN and p50 abundance around the HIV-1 promoter site, with either wtκB or Δκ B, in HIV-1 vector-transduced 293FT cells. *Error bars* indicate ± S.E. **, $p < 0.01$; *NS*, not significant.

noprecipitation assay using α-WRN antibodies (Fig. 2*D*). Upon TNF-α stimulation, the ratio of interactions between WRN and RelA was increased in nuclear fraction, whereas consistent interaction of WRN with p50 through transactivation was observed. Together, these results support that the interaction between WRN and NF-κB occurs in nuclear compartment.

WRN Recruits HIV-1 Promoter via NF-κB Binding Sequence—WRN was previously reported to be tethered on the HIV-1 LTR in the presence of the viral transactivating Tat protein (11). However, the mechanisms underlying WRN recruitment to LTR are still unknown (11). A previous study showed that LTR is occupied by the repressive p50/p50 homodimer in an unstimulated state, which is replaced by the RelA/p50 heterodimer after TNF-α stimulation (15). Thus, the NF-κB dimers possibly help to recruit WRN to the NF-κB-responsive elements on the HIV-1 LTR. To investigate this possibility, we constructed lentiviral vectors with wild-type κB (wtκB) or κB with site-specific mutations (muκB) (28) in the HIV-1 LTR (Fig. 3*A*). These vectors did not encode Tat protein. Further, we established 293FT cells, which were stably transduced with each vector. Using genomic PCR, both cells were confirmed to harbor similar amounts of vector (Fig. 3*B*). The muκB HIV-1 vector completely lost its response to TNF-α-stimulated activation of NF-κB (Fig. 3*C*). By performing ChIP assays on these cells, we found that WRN was recruited to HIV-1 LTR in the absence of Tat (Fig. 3*D*). Furthermore, introduction of κB site mutations in the LTR decreased the amount of WRN as well as p50 on the LTR, suggesting the recruitment of WRN onto the HIV-1 κB sites along with p50 (Fig. 3*D*).

WRN Cooperatively Activates HIV-1 Transcription with RelA/p50 after TNF-α Stimulation—Using 293FT-LTR-Luc5 cells, we further examined whether WRN is co-transcriptionally activated with NF-κB during HIV-1 transcription (27). The 293FT-LTR-Luc5 cell line harbors a copy of luciferase, under the control of an intact HIV-1 LTR with no Tat gene, stably integrated as an HIV-1 vector (Fig. 4*A*). ChIP assay showed that the endogenous WRN is recruited to the HIV-1 promoter in the absence of Tat (Fig. 4*B*). When plasmids encoding shRNA against *WRN* (Fig. 4*C*) were co-transfected into these cells together with plasmids expressing RelA/p50, luciferase activity following RelA/p50 transduction was reduced by WRN knockdown (Fig. 4*D*). In a time-course analysis of the luciferase activity after TNF-α stimulation, it was found that the activity was down-regulated and that the levels of elongated viral transcripts were reduced in the WRN knockdown cells, 30 min after TNF-α stimulation (Fig. 4, *E* and *F*). These results provide evidence for cooperative activation of WRN and RelA/p50-mediated basal viral transcriptional activation.

The Amount of WRN Is Increased on the HIV-1 Promoter via TNF-α Stimulation—The p50/p50 homodimer was previously demonstrated to act as a transcriptional repressor on the HIV-1 LTR in unstimulated cells, functioning via interaction with HDAC1 (15). Upon stimulation-mediated activation of the NF-κB pathway, the homodimer is replaced by RelA/p50 and HDAC1 is detached from the LTR. The difference of recruitment ratio among these proteins is caused by immunoprecipitation capacities of antibodies used. Based on our observations showing the recruitment of WRN to the LTR in unstimulated

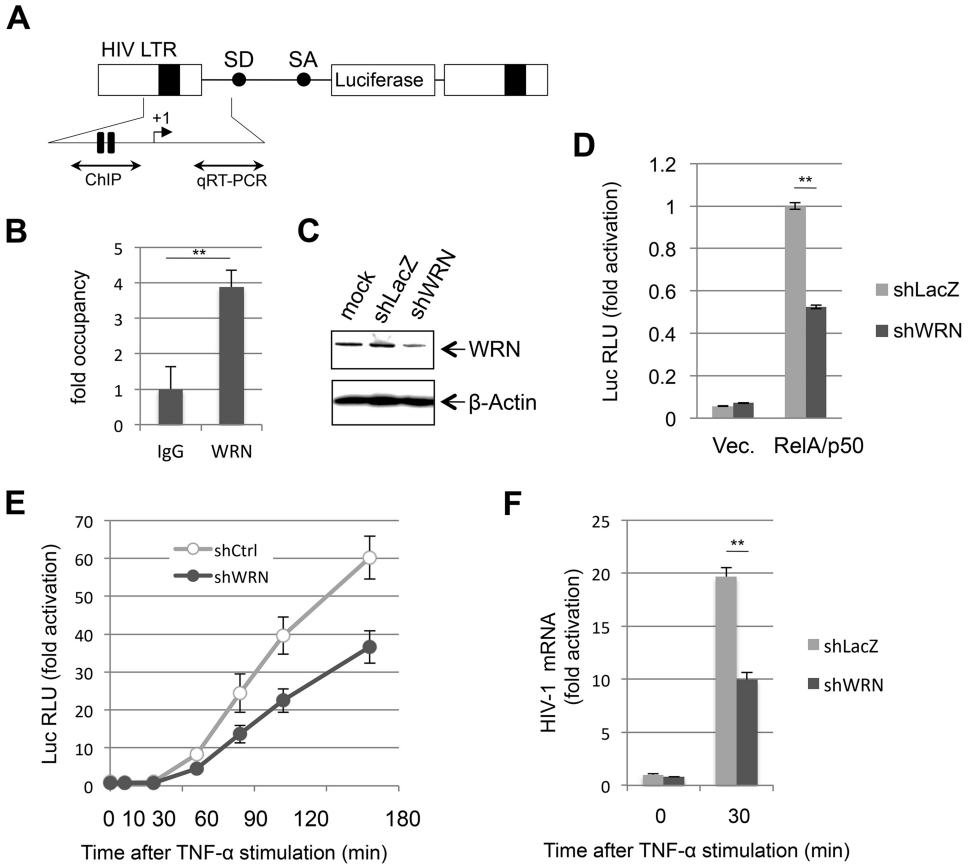

FIGURE 4. **WRN cooperatively activate HIV-1 promoter with RelA/p50.** *A*, a schematic representation of the luciferase expressing HIV-1 vector. *Arrow* indicates PCR region of ChIP or quantitative RT-PCR (*qRT-PCR*). *SD*, Splicing Donner; *SA*, Splicing Acceptor. *B*, ChIP assay of WRN abundance around the HIV-1 promoter site in 293FT-LTR-Luc-5 cells. *C*, expression of WRN and β-actin protein in 293FT-LTR-Luc-5 cells transiently transfected with plasmid expressing sh-WRN (*shWRN*) or control LacZ shRNA (*shLacZ*). *D*, cells were co-transfected with vectors (*Vec.*) expressing NF-κB subunits and shWRN or with respective control vectors in various combinations. Luciferase activity was measured 48 h after transfection and normalized using control plasmid transfectants. *Luc RLU*, luciferase relative light units. *E* and *F*, luciferase activity in sh-control (*shCtrl*)- or shWRN-transduced 293FT-LTR-Luc-5 cells. 48 h after transfection, transfected cells were stimulated by 10 ng/ml TNF-α for the indicated time, and luciferase activity was measured (*E*). shRNA-transfected cells were treated with 10 ng/ml TNF-α for 30 min, and HIV-1 vector transcripts were measured by quantitative RT-PCR. These levels were normalized to individual endogenous GAPDH mRNA (*F*). *Error bars* indicate ± S.E. **, $p < 0.01$.

cells (Figs. 3*D* and 4*B*) and interaction between WRN and RelA after stimulation (Fig. 2, *B* and *D*), WRN appears to function as a coactivator of RelA/p50 and possibly exists around the LTR as a HDAC1 competitor. To confirm this theory, we transfected 293FT-LTR-Luc5 cells with plasmids expressing shRNA against *WRN* (Fig. 5*A*) and performed ChIP assay using these cells. Depletion of WRN from the HIV-1 promoter did not affect the amount of HDAC1 or p50 on the HIV-1 LTR in an unstimulated state (Fig. 5*B*). In contrast, the recruitment of WRN, RelA, and p50 was accompanied by a detachment of HDAC1 from the HIV-1 LTR after TNF-α stimulation (Fig. 5*C*). These results suggest that the function of WRN in transcriptional regulation is mediated via NF-κB activation, rather than through a change in histone modification pattern in the unstimulated state.

WRN Recruits κB Site on IL-8 Promoter and Enhances Its Transcriptional Activation—Because NF-κB plays an important role in transcriptional regulation during an inflammatory response, WRN is possibly recruited to endogenous gene promoters, which are under the control of NF-κB. Based on this hypothesis, we analyzed the promoter region of IL-8, an NF-κB-responsive gene, which promotes cancer development through cell migration and angiogenesis (Fig. 6*A*) (30, 31). Here, WRN was found to exist on the IL-8 promoter prior to stimulation (Fig. 6*B*). Furthermore, following TNF-α stimulation, we observed an increased recruitment of WRN and RelA/p50 on the IL-8 promoter region (Fig. 6*B*), suggesting a possible RelA/p50-mediated involvement of WRN in transcriptional regulation of IL-8. Because knockdown of WRN decreased TNF-α-mediated activation of *IL-8* mRNA expression (Fig. 6, *C* and *D*), our data indicate that WRN positively regulates the stimulation-mediated *IL-8* gene expression along with the recruitment of RelA/p50 to the IL-8 promoter region.

Discussion

WRN is a multifunctional protein that interacts with host proteins and participates in various steps of DNA transactivation such as replication, recombination, repair of breaks, and telomere maintenance (4). However, the participation of WRN in cellular transcriptional regulation is still poorly understood. In this study, we observed the involvement of WRN in transcriptional regulation of certain genes, which are under the control of NF-κB. WRN interacted physically with p50 and RelA via the RQC domain and was recruited, in an NF-κB

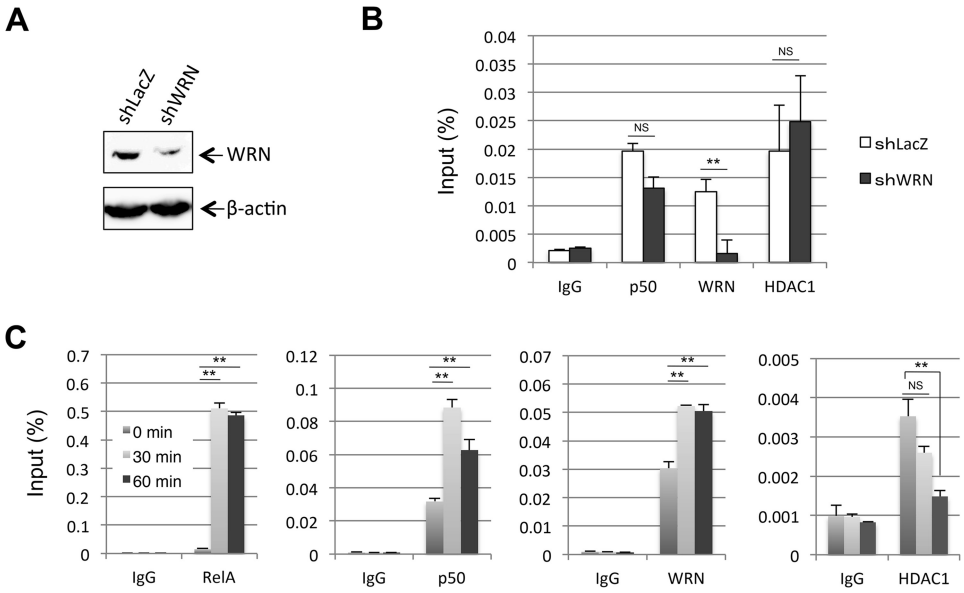

FIGURE 5. **WRN is recruited to the HIV-1 promoter in stimulation-dependent manner.** *A* and *B*, ChIP analysis of p50, WRN, and HDAC1 levels at the NF-κB site on U3 region in the HIV-1 promoter in steady states in shLacZ (control)- or shWRN-transduced cells. *A*, expression of WRN and β-actin protein in 293FT-LTR-Luc-5 cells transiently transfected with plasmid expressing sh-WRN (*shWRN*) or control LacZ shRNA (*shLacZ*). *B*, the abundance of indicated proteins exists at the NF-κB site on the HIV-1 promoter in 293FT-LTR-Luc-5 cells. *C*, ChIP analysis of RelA, p50, WRN, and HDAC1 levels at the NF-κB site on U3 region in the HIV-1 promoter upon TNF-α stimulation. Cells were harvested at 0, 30, and 60 min after TNF-α stimulation. Results presented here are the average of at least two independent experiments. *Error bars* indicate ± S.E. *ND*, not detectable; **, $p < 0.01$; *NS*, not significant.

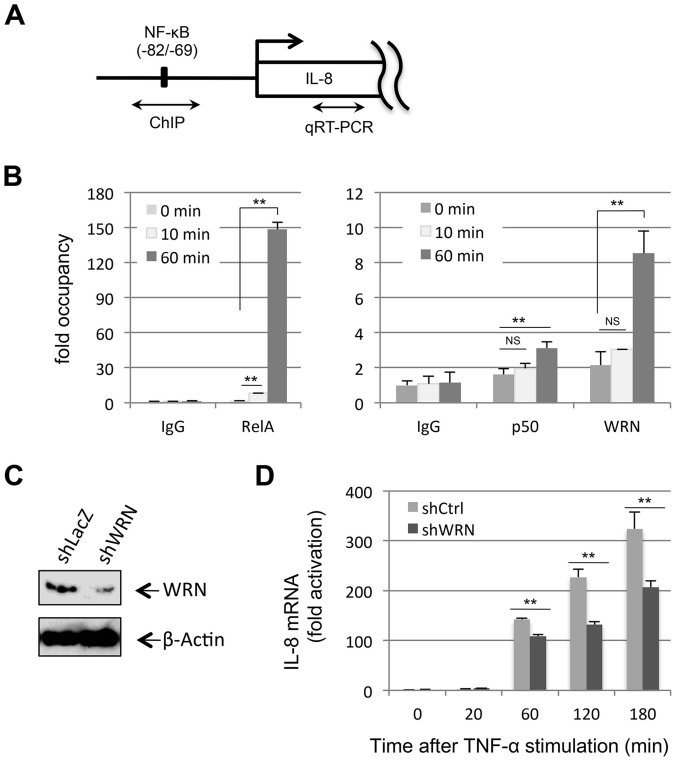

FIGURE 6. **WRN recruits to IL-8 promoter and play a positive role in IL-8 transcription.** *A*, a schematic representation around IL-8 promoter region. *Arrow* indicates PCR region of ChIP or quantitative RT-PCR. *B*, ChIP analysis of RelA p50, WRN, and HDAC1 levels at the NF-κB site in IL-8 promoter upon TNF-α stimulation in 293FT cells. *C*, expression of WRN and β-actin protein in 293FT cells after 48 h transfection with plasmid expressing shWRN (*shWRN*) or control LacZ shRNA (*shLacZ*). *D*, shRNA-transfected cells were treated with 10 ng/ml TNF-α for the indicated time, and *IL-8* transcripts were measured by quantitative RT-PCR. These levels were normalized to individual endogenous GAPDH mRNA. *shCtrl*, sh-control. *Error bars* indicate ± S.E. **, $p < 0.01$; NS, not significant.

binding-dependent manner, to the HIV-1 LTR and the endogenous IL-8 promoter. Because the RQC domain is also present in other members of the human RecQ family (*e.g.* BLM, RecQ1, and RecQ5β), a possible involvement of these proteins in transcriptional regulation can be suggested.

The HIV-1 LTR contains several cis-regulating elements including sequences that recruit host transcription factors, such as NF-κB and SP1. Among them, NF-κB plays a major role in Tat-independent regulation of the integrated LTR either in a positive or in a negative manner depending on the presence or absence of signals necessary for activating the NF-κB pathway. In an unstimulated state, the LTR is occupied by the suppressive p50/p50 homodimer, with which WRN was found to be recruited. We confirmed that this recruitment of WRN is NF-κB-dependent and showed a direct interaction of WRN with p50. This was accompanied by a decrease in the association of WRN with the LTR upon introduction of site-specific mutations in the NF-κB sites. However, there is a realistic possibility of involvement of other transcriptional factors based on the finding that WRN existed on the mutated LTR, albeit at a decreased level.

It was previously reported that the p50/p50 homodimer acts as a transcriptional suppressor of the HIV-1 LTR by being deficient in TAD domains and also by recruiting HDAC1 in unstimulated cells (15). Despite the p50/p50-dependent recruitment to the unstimulated LTR (Figs. 3*D* and 4*B*), WRN enhanced the RelA/p50-mediated transactivation of the LTR (Fig. 4, *D–F*). These data supported the idea of competitive recruitment of WRN and HDAC1. However, the knockdown of WRN did not affect the association of HDAC1 with HIV-1 LTR (Fig. 5*B*). Thus, the function of WRN in transcriptional regulation is mediated through the activation of NF-κB rather than through a change in the histone modification pattern in the unstimulated state.

We determined that the RHD domain in RelA and p50 is responsible for their interaction with the WRN protein (Fig. 1*E*). However, we observed no interaction of WRN with the p105 protein, the p50 precursor (Fig. 2*D*), although the RHD domain is also contained in p105. This may be due to intramolecular masking of the nuclear localization signal in the RHD domain in the p105 protein (32), which possibly blocks the interaction with WRN. Similarly, we observed the stimulation-dependent interaction of WRN with RelA by the immunoprecipitation analysis not only using the nuclear extract (Fig. 2*D*) but also using the whole cell lysate (Fig. 2*B*). This may also result from the masking of the nuclear localization signal in RHD domain in RelA by the inhibitory IκB protein in unstimulated status (14). Taken together, the association between WRN and NF-κB occurs in the nucleus possibly because of the reorganization of WRN of the active form of NF-κB, as well as the constitutive localization of WRN in the nucleus.

For immortalized $WRN^{-/-}$ WS fibroblasts, Sharma *et al.* (11) concluded that WRN participates in the recruitment of PCAF/P-TEFb-containing transcription complexes to HIV-1 LTR. Taken together with their findings, the RelA/p50 activation-mediated increase in association of WRN to the LTR is expected to result in enhanced recruitment of the PCAF/P-TEFb-containing transcription complexes. This idea was supported by the observation that an increased recruitment of WRN was accompanied by the withdrawal of HDAC1 (Fig. 5*C*).

WS causes premature aging accompanied by rare cancers (1, 2). Nowadays, several studies have shown that NF-κB plays an important role in maintaining cellular senescence in normal cells. There is growing interest in dysfunction of NF-κB response as a potential trigger of aging-related disease (33, 34). Wang *et al.* (35) showed that when compared with RelA intact murine fibroblasts, RelA knock-out murine fibroblasts became immortalized at a faster rate after several passages, and that the rate of abnormal chromosomal structure was increased in RelA knock-out murine fibroblasts. A gene array study showed that the knockdown of WRN protein levels in normal human fibroblasts affected certain biological pathways including the NF-κB pathway (10). Together these results imply that the loss of normal NF-κB function leads to collapse of cellular senescence in normal cells. For this reason, the functional relationship between WRN and NF-κB is crucial in maintaining cellular senescence.

There has been increasing evidence concerning an active cross-talk between senescent and neighboring cells via secretory factors such as growth factors, extracellular proteases, cytokines, and chemokines (19). These phenomena are collectively known as SASP (20, 21). IL-6 and IL-8, which are transcriptionally regulated by NF-κB, are major SASP proteins (22). Our ChIP analysis indicated the recruitment of WRN at endogenous IL-8 promoters accompanied by an increased ratio of RelA/p50 recruitment to IL-8 promoter and positive regulation of the IL-8 transcription after TNF-α stimulation. Possibly, the loss of WRN led to an irregular secretion of the SASP proteins, which control normal cell senescence and aging. Overall, it is likely that the normal function of NF-κB may be partially hampered by the loss of WRN, and the accumulation of transcriptional disadvantages could cause premature aging disorders and lead to the development of cancer.

In conclusion, we show that the DNA helicase WRN in cooperation with RelA/p50 plays a role in RNAP II-dependent transcriptional activation. This study contributes to the understanding of WRN transcriptional activity, which could have deeper implications in elucidating the mechanisms involved in both cellular senescence and maintenance of homeostasis. Further studies are required to examine whether WRN regulates other NF-κB-responsive genes and to identify the proteins contained in the WRN-RelA/p50 complexes. These studies will help to understand the role of WRN as a transcriptional regulator for cellular homeostasis and may clarify important biological events underlying WRN deficiency diseases *in vivo*.

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