The Werner Syndrome Helicase Is a Cofactor for HIV-1 Long Terminal Repeat Transactivation and Retroviral Replication*

The Werner syndrome helicase (WRN) participates in DNA replication, double strand break repair, telomere maintenance, and p53 activation. Mutations of *wrn* cause Werner syndrome (WS), an autosomal recessive premature aging disorder associated with cancer predisposition, atherosclerosis, and other aging related symptoms. Here, we report that WRN is a novel cofactor for HIV-1 replication. Immortalized human WRN**/−**/−WS fibroblasts, lacking a functional *wrn* gene, are impaired for basal and Tat-activated HIV-1 transcription. Overexpression of wild-type WRN transactivates the HIV-1 long terminal repeat (LTR) in the absence of Tat, and WRN cooperates with Tat to promote high-level LTR transactivation. Ectopic WRN induces HIV-1 p24Gag production and retroviral replication in HIV-1-infected H9 lymphocytes. A dominant-negative helicase-minus mutant, WRNK577M, inhibits LTR transactivation and HIV-1 replication. Inhibition of endogenous WRN, through co-expression of WRNK577M, diminishes recruitment of p300/CREB-binding protein-associated factor (PCAF) and positive transcription elongation factor b (P-TEFb) to Tat/transactivation response-RNA complexes, and immortalized WS fibroblasts exhibit comparable defects in recruitment of PCAF and P-TEFb to the HIV-1 LTR. Our results demonstrate that WRN is a novel cellular cofactor for HIV-1 replication and suggest that the WRN helicase participates in the recruitment of PCAF/P-TEFb-containing transcription complexes. WRN may be a plausible target for antiretroviral therapy.

Several lines of investigation allude to a role for the WRN protein and RecQ DNA helicases in transcription. Balajee et al. (1) have demonstrated that WRN contributes to general RNA pol II-dependent transcription, although its mechanism remains unclear. Interestingly, these authors found that a 27-amino acid direct-repeat sequence strongly activated transcription in yeast two-hybrid experiments, independent of WRN 3′ → 5′ DNA helicase activity (1) (Fig. 1A) suggesting that WRN interacts with cellular factors to modulate RNA pol II-dependent transcription. The WRN protein localizes to nucleoli and the nucleoplasm of transcriptionally active cells (1, 2). Moreover, Laine et al. (3) have shown that WRN stimulates topoisomerase I DNA-unwinding activity that could influence cellular transcription. The yeast WRN homologue, SGS1, also participates in DNA replication and RNA pol I-dependent transcription (4), and the WRN helicase enhances RNA pol I-dependent transcription of ribosomal RNA (5).

In the present study, we have investigated whether WRN contributes to HIV-1 LTR transactivation and retroviral replication. The HIV-1 LTR contains upstream enhancer elements (e.g. NF-κB and SP1) that synergize with the transactivator protein, Tat, bound to TAR-RNA, to promote retroviral gene expression in HIV-1-infected tissues, macrophages/monoocytes, and CD4+ T-lymphocytes (6–17). The mechanism by which Tat/TAR-RNA complexes regulate transcription from the HIV-1 LTR involves the concerted recruitment of a plethora of cellular factors, including p300/CREB-binding protein (p300/CBP) (18–25), PCAF/hGCN5 (20–22, 26–30), P-TEFb (30–33), SET7/SET9 methyltransferases (34), SIRT1 (35), the Brm component of the SWI/SNF chromatin-remodeling complex (36–38), and other factors (39, 40). The abbreviations used are: pol II, polymerase II; AIDS, acquired immune deficiency syndrome; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; HIV-1, human immunodeficiency virus type-1; LTR, long terminal repeat; PCAF, p300/CREB-binding protein-associated factor; P-TEFb, positive transcription elongation factor b; TAR-RNA, transactivation response-ribonucleic acid; WRN, Werner syndrome helicase; WS, Werner syndrome; PHA, phytohemagglutinin; CREB, cAMP-response element-binding protein; CMV, cytomegalovirus; RSV, Rous sarcoma virus; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; hIL-2, human interleukin 2; CBP, CREB-binding protein.

The Werner syndrome helicase (WRN) participates in DNA replication, double strand break repair, telomere maintenance, and p53 activation. Mutations of *wrn* cause Werner syndrome (WS), an autosomal recessive premature aging disorder associated with cancer predisposition, atherosclerosis, and other aging related symptoms. Here, we report that WRN is a novel cofactor for HIV-1 replication. Immortalized human WRN**/−**/−WS fibroblasts, lacking a functional *wrn* gene, are impaired for basal and Tat-activated HIV-1 transcription. Overexpression of wild-type WRN transactivates the HIV-1 long terminal repeat (LTR) in the absence of Tat, and WRN cooperates with Tat to promote high-level LTR transactivation. Ectopic WRN induces HIV-1 p24Gag production and retroviral replication in HIV-1-infected H9 lymphocytes. A dominant-negative helicase-minus mutant, WRNK577M, inhibits LTR transactivation and HIV-1 replication. Inhibition of endogenous WRN, through co-expression of WRNK577M, diminishes recruitment of p300/CREB-binding protein-associated factor (PCAF) and positive transcription elongation factor b (P-TEFb) to Tat/transactivation response-RNA complexes, and immortalized WS fibroblasts exhibit comparable defects in recruitment of PCAF and P-TEFb to the HIV-1 LTR. Our results demonstrate that WRN is a novel cellular cofactor for HIV-1 replication and suggest that the WRN helicase participates in the recruitment of PCAF/P-TEFb-containing transcription complexes. WRN may be a plausible target for antiretroviral therapy.

The Abbreviations used are: pol II, polymerase II; AIDS, acquired immune deficiency syndrome; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; HIV-1, human immunodeficiency virus type-1; LTR, long terminal repeat; PCAF, p300/CREB-binding protein-associated factor; P-TEFb, positive transcription elongation factor b; TAR-RNA, transactivation response-ribonucleic acid; WRN, Werner syndrome helicase; WS, Werner syndrome; PHA, phytohemagglutinin; CREB, cAMP-response element-binding protein; CMV, cytomegalovirus; RSV, Rous sarcoma virus; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; hIL-2, human interleukin 2; CBP, CREB-binding protein.
plex (36), and the splicing factor, SKIP (37). The Tat protein is acetylated on Lys\(^{28}\), Lys\(^{50}\), and Lys\(^{51}\) by the transcriptional coactivators/acyltransferases, p300/CBP and PCAF/hGcn5 (20–22, 24, 27–30, 38), which has been shown to modulate Tat interactions with P-TEFb and BRm, as well as the ability of Tat for binding TAR-RNA (20, 30, 35, 36, 39). The formation of Tat/TAR-RNA/P-TEFb/PCAF complexes on the HIV-1 LTR stimulates Ser\(^{2}\)-Ser\(^{5}\)-phosphorylation of the RNA pol II carboxyl-terminal domain associated with increased transcriptional elongation (30, 37, 40). Importantly, our results demonstrate that the WRN helicase interacts and cooperates with Tat to transactivate the HIV-1 LTR to promote retroviral replication through the stable recruitment of PCAF/P-TEFb to Tat/TAR-RNA transcription complexes.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Transfection**—pCV126 RSV-HIV-1 Tat (exon 1: amino acids 1–72, FLAG-tagged), pCV122 RSV-HIV-1 Tat\(_{K28A/K50A}^{28}\) pEV284 HIV-1 LTR-luciferase (contains pBR322 nucleotides 345–531), and pGEX-GST-HIV-1 Tat HA (HA) and pGEX-GST-HIV-1 Tat\(_{K28A/K50A}^{28}\) (HA) plasmids were described by Kiernan **et al.** (20). pCV63 HIV-1 LTR TAR-luciferase is deleted for the 3-nucleotide bulge within TAR-RNA; and the episomal pCV745 HIV-1 LTR-luciferase construct used in ChIP analyses contains an Xhol-BamHI fragment that spans the HIV-1 LTR. CMV-wild type-WRN and CMV-WRN\(_{K577M}^{377}\) expression constructs have been described in Moser **et al.** (41). All transfections were performed using Lipofectamine (Invitrogen) or Superfect (Qiagen) reagents.

**Cell Culture and Concentration of Retrovirus Particles**—SV40-immortalized human WRN\(^{-/-}\) WS fibroblasts (41), 293A fibroblasts (Quantum Biotechnology), and HeLa cells (ATCC, CCL-2) were grown in Dulbecco’s modified Eagle’s medium (ATCC) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin, and 100 \(\mu\)/ml streptomycin sulfate (Invitrogen) and cultured at 37 °C and 5% CO\(_{2}\). HuT-78 cells (ATCC, TIB-161) were grown in RPMI 1640 medium (ATCC) supplemented with 20% fetal bovine serum, and 100 \(\mu\)/ml penicillin, 100 \(\mu\)/ml streptomycin sulfate, and 20 \(\mu\)/ml gentamycin sulfate (Sigma Corp.), and cultured at 37 °C and 10% CO\(_{2}\). The HIV-1-infected H\(_{9}^{9}\)W\(_{11002}^{110}\) fibroblasts exhibited reduced LTR-dependent transactivation and the production of infectious viral particles, HIV-1-infected H\(_{9}^{9}\)W\(_{11002}^{110}\) lymphocytes were stimulated with 50 units/ml recombinant human interleukin-2 (hIL-2; Invitrogen) and phytohemagglutinin (10 \(\mu\)/ml, Sigma) for 72–96 h. Cells were pelleted by centrifugation at 1,500 \(\times\) g for 7 min at 4 °C and the supernatants were filtered through a 0.45-\(\mu\)m filter and layered upon a 70% sucrose, Tris-HCl, pH 7.4, cushion. HIV-1 particles were concentrated by ultracentrifugation at 44,000 \(\times\) g for 24 h at 4 °C using a Beckman 70.1 Ti rotor and a model L8–70 M ultracentrifuge.

**Chromatin Immunoprecipitation**—3 \(\times\) 10\(^{6}\) HeLa cells or WRN\(^{-/-}\) WS fibroblasts were co-transfected with an episomal HIV-1 LTR-luciferase (pCV745) plasmid and RSV-HIV-1 Tat using Superfect reagent (Qiagen). CMV-WRN\(_{K577M}^{377}\) was co-transfected in certain experiments to inhibit the endogenous WRN helicase (41, 42). Chromatin immunoprecipitations were performed by incubating precleared lysates with 40 \(\mu\)/l of protein G-agarose/sheared salmon sperm DNA (Upstate Biotechnology) and 5 \(\mu\)/l of rabbit polyclonal anti-HIV-1 Tat (Advanced Bioscience Laboratories), goat polyclonal anti-WRN (Santa Cruz Biotechnology), monoclonal anti-p300 (Upstate Biotechnology), goat polyclonal anti-PCAF (Santa Cruz Biotechnology), goat polyclonal anti-cyclin T1 (Santa Cruz Biotechnology), or nonspecific rabbit IgG. Oligonucleosomal DNA fragments spanning the Nuc-1 position of the HIV-1 LTR were amplified by PCR using oligonucleotide DNA primers that anneal within nucleotides −92 to +180 (40).

**Immunostaining and Laser Confocal Microscopy**—Co-localization between HIV-1 Tat and WRN proteins was visualized by performing immunofluorescence laser confocal microscopy on post-mortem central nervous system tissue samples (thalamus and basal ganglia) from five donor NeuroAIDS patients (7674, 7996, 11406, 13689, and 14433) with primary HIV-1 encephalopathies and one uninfected control brain (3026). Nonspecific antigens were blocked by incubating slides in 3% (w/v) bovine serum albumin, 0.5% Tween 20 in phosphate-buffered saline for 1 h at room temperature. The slides were then immunostained using rabbit polyclonal anti-HIV-1 Tat (Advanced Bioscience Laboratories) and goat polyclonal anti-WRN (Santa Cruz Biotechnology) primary antibodies diluted 1:2000 in BLOTTO buffer (50 mM Tris-HCl, pH 8.0, 2 mM CaCl\(_{2}\), 80 mM NaCl, 0.2% (v/v) IGEpal CA-630, 0.02% (v/v) sodium azide, 5% (v/v) nonfat dry milk) and incubated for 2 h at room temperature with gentle agitation. Samples were washed 2 times for 10 min with BLOTTO buffer and then incubated with rhodamine red-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-goat secondary antibodies (Jackson Laboratories) for 1 h at room temperature. Laser confocal microscopy and three-dimensional Z-stack composite images were generated using a Nikon TE2000-U inverted microscope/Cl confocal system equipped with dual helium-neon lasers. HIV-1 p24\(_{Gag}\) protein expression was visualized in PHA/hIL-2-stimulated HIV-1-infected H\(_{9}^{9}\)W\(_{11002}^{110}\) lymphocytes by immunostaining with a mouse monoclonal anti-HIV-1 p24\(_{Gag}\) primary antibody (Advanced Bioscience Laboratories) and rhodamine red-conjugated anti-mouse secondary antibody (Jackson Laboratories).

**RESULTS**

WS Fibroblasts, lacking a functional wrn gene, are impaired for HIV-1 LTR transactivation. To determine whether WRN contributes to HIV-1 LTR transactivation, immortalized human WRN\(^{-/-}\) WS fibroblasts derived from a Werner syndrome patient (41) and HeLa cells were co-transfected with an HIV-1 LTR-luciferase (firefly) reporter plasmid in the absence or presence of increasing amounts of HIV-1 Tat or a transcriptionally inactive Tat mutant, Tat\(_{K28A/K50A}^{28}\) (20, 21). A tk-\textit{Renilla} luciferase construct was co-transfected to normalize for transfection efficiencies or differences in general transcriptional levels (1) between WRN\(^{-/-}\) WS fibroblasts and HeLa, which contain endogenous WRN. Significant basal HIV-1 LTR-dependent transcription was measured in HeLa cells, whereas WRN\(^{-/-}\) WS fibroblasts exhibited reduced LTR-dependent...
transcription (Fig. 1B). Tat activated the HIV-1 LTR approximately 20-fold in HeLa cells (Fig. 1B). Surprisingly, human WRN−/− WS fibroblasts were severely impaired for Tat-activated HIV-1 LTR-dependent transcription (Fig. 1B). Results from a representative experiment are shown in Fig. 1B. The WRN helicase has been shown to exist in multimeric protein complexes (43) and the dominant-negative helicase-minus mutant, WRNK577M, interferes with endogenous WRN functions (42). Therefore, we co-transfected HeLa cells with CMV-WRNK577M together with HIV-1 LTR-luciferase and RSV-HIV-1 Tat, to determine whether trans inhibition of WRN might block Tat-dependent LTR transactivation. Results in Fig. 1B demonstrate that increasing CMV-WRNK577M inhibited Tat-dependent HIV-1 LTR transactivation in a dose-dependent manner. We next co-transfected immortalized human WRN−/− WS fibroblasts with HIV-1 LTR-luciferase and RSV-HIV-1 Tat in the presence of increasing CMV-wild type-WRN or a pcDNA3.1-GFP control. As the WRN−/− WS fibroblasts express a proteolytically unstable, truncated WRN mutant protein, it was necessary to treat cultures with a cell-permeable 20 μM proteasome inhibitor, lactacystin (10 μM), to stabilize the ectopically expressed wild type WRN (Myc-tagged) protein. CMV-wild type-WRN alone weakly transactivated the HIV-1 LTR (approximately 5-fold) in the absence of Tat (Fig. 1C). Increasing CMV-wild type-WRN significantly restored Tat-dependent LTR transactivation (Fig. 1C). The expression of HIV-1 Tat and WRN (Myc-tagged) proteins was detected by immunofluorescence microscopy (Fig. 1D). Collectively, these findings indicate that WRN helicase functions are essential for HIV-1 transcription and retroviral replication.

The WRN helicase cooperates with Tat to transactivate the HIV-1 LTR. We next tested the effects of overexpressing wild type WRN upon basal and Tat-activated HIV-1 LTR-dependent transcription by co-transfecting HuT-78 lymphocytes and HeLa cells with HIV-1 LTR-luciferase and RSV-HIV-1 Tat in the absence or presence of CMV-wild type-WRN, CMV-WRNK577M or CβS vector control. Tat transactivated the HIV-1 LTR approximately 70-fold in HuT-78 cells (Fig. 2A). Overexpression of wild type WRN alone transactivated the HIV-1 LTR approximately 14-fold (Fig. 2A). Increasing amounts of CMV-wild type-WRN also resulted in higher Tat-dependent LTR transactivation (compare 70-fold for Tat alone to 217-fold for wild type WRN + Tat). By contrast, the helicase-minus WRNK577M mutant inhibited Tat-dependent LTR transactivation in a dose-dependent manner. The CβS control did not affect Tat-dependent LTR transactivation (Fig. 2A). Similar results were obtained using HeLa cells (Fig. 2B). CMV-wild type-WRN alone transactivated the HIV-1 LTR approximately 6-fold, whereas the WRNK577M mutant repressed basal LTR-dependent transcription (Fig. 2B). Increasing amounts of CMV-wild type-WRN resulted in increased Tat-dependent LTR transactivation (compare 37-fold for Tat alone to 72-fold for wild type WRN + Tat). The helicase-minus WRNK577M mutant markedly inhibited Tat-dependent LTR transactivation (from 37 - to approximately 3-fold). The CβS vector did not affect Tat-dependent LTR transactivation (Fig. 2B). Neither overexpression of wild type WRN nor WRNK577M altered HIV-1 Tat protein expression (Fig. 2C). Another related DNA helicase, BLM-associated with Bloom syndrome (44), which has been shown to interact with WRN (44, 45) did not influence basal or Tat-activated HIV-1 LTR-dependent transcription (data not shown) indicative that effects of WRN upon HIV-1 LTR transactivation are independent of WRN-BLM interactions.

We then investigated whether the effects of WRN upon HIV-1 transcription require the TAR element and Tat/TAR-RNA interactions. Tat binds to a uracil-containing trinucleotide bulge within TAR-RNA and stimulates transcriptional elongation by recruiting cellular factors (e.g. P-TEFb) to the HIV-1 LTR (see diagram, Fig. 2E) (30–33, 46, 47). HeLa cells were co-transfected with a mutant HIV-1 LTR TAR-luciferase reporter plasmid, deleted for the 3-nucleotide bulge within TAR-RNA, and RSV-HIV-1 Tat in the absence or presence of increasing CMV-wild type-WRN or CMV-WRNK577M. Tat only weakly transactivated the HIV-1 LTR TAR-luciferase construct (approximately 2.6-fold, Fig. 2D). Neither ectopic wild type WRN nor the WRNK577M mutant significantly altered basal or Tat-activated transcription from the HIV-1 LTR TAR (Fig. 2D). These results indicate that effects of WRN upon HIV-1 LTR-dependent transcription require an intact TAR-RNA element.

WRN is essential for the recruitment of PCAF/P-TEFb transcription complexes to the HIV-1 LTR. To determine whether the effects of WRN upon LTR transactivation reflect direct binding or altered recruitment of transcriptional coactivator/ acetyltransferase proteins to the HIV-1 LTR, we performed LTR-ChIP experiments. Tat interacts with the coactivators, p300/CREB-binding protein and PCAF, on the HIV-1 LTR and these factors acetylate Tat on lysine residues (Lys28, Lys50, and Lys51), which modulates TAR-RNA binding and interactions with other cofactors, such as P-TEFb (cyclin T1-cdk9) (18–22, 30, 39). Balaje et al. (1) have shown that WRN contributes to RNA pol II transcription in a helicase-dependent and helicase-independent manner. To test whether WRN influences the recruitment of cellular factors to the HIV-1 LTR, HeLa cells and immortalized human WRN−/− WS fibroblasts were co-transfected with an episomal HIV-1 LTR-luciferase construct and RSV-HIV-1 Tat. CMV-WRNK577M was co-transfected in certain experiments to inhibit endogenous WRN functions. ChIPs were performed to detect nucleoprotein interactions on the HIV-1 LTR at the Nuc-1 position (~92 to +180) in the vicinity of Tat/TAR-RNA (Fig. 2E) (40). These results demonstrate that Tat and cellular cofactors, p300, PCAF, cyclin T1, and WRN, were recruited to the episomal HIV-1 LTR in transfected HeLa cells (Fig. 2F). Importantly, inhibition of endogenous WRN, through co-expression of the helicase-minus WRNK577M mutant, abrogated recruitment of WRN to Tat-containing LTR complexes and diminished recruitment of PCAF and cyclin T1 (Fig. 2F). Tat and p300 interactions on the HIV-1 LTR were not affected by WRNK577M. We observed similar impairment in the recruitment of PCAF and cyclin T1 to Tat-containing LTR complexes in transfected immortalized WRN−/− WS fibroblasts (Fig. 2F). Furthermore, we demonstrated that WRN interacts with purified recombinant GST-HIV-1 Tat and the transcriptionally inactive GST-HIV-1 TatC28A/K50A mutant in biochemical GST pull-down experiments (Fig. 2G). Our findings suggest that WRN is an essential cofactor for HIV-1 tran-
FIGURE 1. Immortalized human WRN<sup>−/−</sup> WS fibroblasts are impaired for HIV-1 LTR transcriptional activation. A, diagram of the WRN. The 3′ → 5′ exonuclease and helicase domains are shown (NLS, nuclear localization sequence). Two mutations are labeled in bold: E84A, an exonuclease inactivating mutation, and K577M, a dominant-negative mutation located within the ATPase site of the 3′ → 5′ DNA helicase domain (1, 41, 42). B, immortalized human WRN<sup>−/−</sup> WS fibroblasts (41) were co-transfected with tk-renilla-luciferase and HIV-1 LTR-luciferase (firefly) reporter plasmids (0.5 μg each) in the presence of increasing amounts of RSV-HIV-1 Tat or RSV-HIV-1 Tat<sub>E84A/K577M</sub> (0.15, 0.25, and 0.5 μg) (20). CMV-WRN<sub>K577M</sub> (42) was co-transfected (0.5 and 1.0 μg) in certain experiments (asterisks) to block endogenous WRN functions and inhibit Tat-dependent HIV-1 LTR trans-activation. Samples were normalized to yield approximately equivalent Renilla luciferase expression to control for transfection efficiencies and any differences in overall transcriptional levels between WRN<sup>−/−</sup> WS fibroblasts and HeLa cells (1). Results from a representative experiment are shown (n = 3). C, expression of wild-type WRN partially counters the HIV-1 transcriptional impairment in transfected human WRN<sup>−/−</sup> fibroblasts. Immortalized human WRN<sup>−/−</sup> WS fibroblasts were co-transfected with HIV-1 LTR-luciferase and tk-renilla-luciferase plasmids in the presence or absence of RSV-HIV-1 Tat (0.5 μg) and/or CMV-WRN<sup>−/−</sup> (wild-type) or pcDNA3.1-GFP expression constructs (0.25, 0.5, and 1.0 μg). Error bars representative of standard deviations (n = 2). D, HIV-1 Tat and WRN (Myc-tagged) proteins were detected in transfected WRN<sup>−/−</sup> WS fibroblasts by immunofluorescence microscopy.
The WRN helicase strongly co-localized with HIV-1 Tat in central nervous system tissue sections (thalamus and basal ganglia) from donor HIV-1-infected NeuroAIDS patients with primary viral encephalopathies (Fig. 3A). Laser confocal microscopy was performed on five patient samples and one uninfected control brain (data not shown) that were immunostained with anti-HIV-1 Tat (red) and anti-WRN (green) primary antibodies and appropriate fluorescent secondary antibodies. A representative three-dimensional Z-stack composite image from a
Recruitment of WRN to HIV-1 Tat/P-TEFb Complexes

A

HIV-1 Tat
Merge

WRN

single patient (11406) is shown in Fig. 3A. Significant co-localization was observed in merged images, consistent with results in Fig. 2G demonstrating that GST-HIV-1 Tat and WRN interact in vitro.

WRN modulates LTR transactivation and retroviral replication in HIV-1-infected T-lymphocytes. To determine whether ectopic WRN or the helicase-minus WRNK577M mutant influences HIV-1 replication, the virus-producing HIV-1-infected H9HIV-1IIIB lymphoid cell line, H9HIV-1IIIB (ATCC number CRL-8543, Ref. 48), was transfected with increasing amounts (1.0 and 3.0 μg) of CMV-wild type-WRN, CMV-WRNK577M, or pcDNA3.1-GFP control and, following 48 h, the cultures were stimulated by treatment with PHA (10 ng/ml) and recombinant hIL-2 (50 units/ml) to induce high level viral replication. After 72 h, cells were harvested by centrifugation and intracellular HIV-1 p24Gag production was observed by immunoblotting. Relative actin protein levels are provided as a control for equivalent loading. Increased intracellular HIV-1 p24Gag production was observed with increasing CMV-wild type-WRN, whereas the dominant-negative WRNK577M mutant resulted in reduced HIV-1 replication. Uninfected Jurkat and untransfected PHA/hIL-2-stimulated H9HIV-1IIIB lymphocytes are shown as controls.

B

H9HIV-1IIIB

Actin

Uninfected

Jurkat

WRN

WRNK577M

GFP

transfected cells were determined by direct fluorescence microscopy (inset micrographs) and the supernatants were collected and extracellular HIV-1 particles were concentrated by ultracentrifugation at 44,000 × g at 4°C for 24 h. Relative amounts of extracellular HIV-1 p24Gag protein were determined by immunoblotting. Uninfected HuT-78 lymphocytes were included as a negative control. Relative percentages of extracellular HIV-1 p24Gag protein produced by transfected HIV-1-infected H9HIV-1IIIB lymphocytes are expressed as: 100 − (ΔHIV-1 p24Gag (% GFP-positive cells) and are represented by black bars. The cell pellets were lysed by repeated freeze-thawing and relative luciferase activities were measured using equivalent amounts of total cellular proteins.

C

FIGURE 3. Inhibition of endogenous WRN through co-expression of the dominant-negative helicase-minus WRNK577M mutant inhibits HIV-1 replication in PHA/hIL-2-stimulated H9HIV-1IIIB lymphocytes. A, nuclear co-localization between HIV-1 Tat and WRN proteins was observed in central nervous system tissue sections (thalamus and basal ganglia) from donor HIV-1-infected NeuroAIDS patients by immunofluorescence laser confocal microscopy using anti-HIV-1 Tat (red) and anti-WRN (green) primary antibodies. Post-mortem samples from five different HIV-1-infected NeuroAIDS patients and one uninfected control brain (data not shown) were analyzed; a representative three-dimensional Z-stack composite from a single patient (11406) is shown. B, the virus-producing HIV-1-infected lymphoid cell line, H9HIV-1IIIB (ATCC number CRL-8543, Ref. 48), was transfected with increasing amounts (1.0 and 3.0 μg) of CMV-wild type-WRN, CMV-WRNK577M, or pcDNA3.1-GFP control and, following 48 h, the cultures were stimulated by treatment with PHA (10 ng/ml) and recombinant hIL-2 (50 units/ml) to induce high level viral replication. After 72 h, cells were harvested by centrifugation and intracellular HIV-1 p24Gag production was observed by immunoblotting. Relative actin protein levels are provided as a control for equivalent loading. Increased intracellular HIV-1 p24Gag production was observed with increasing CMV-wild type-WRN, whereas the dominant-negative WRNK577M mutant resulted in reduced HIV-1 replication. Uninfected Jurkat and untransfected PHA/hIL-2-stimulated H9HIV-1IIIB lymphocytes are shown as controls. C, the clonal HIV-1-infected H9HIV-1IIIB cell line was co-transfected with pcDNA3.1-GFP (1.0 μg), HIV-1 LTR-luc (1.0 μg), and increasing amounts (1.0 and 3.0 μg) of CMV-WRNK577M or empty C/S vector control. After 48 h, the transfected cells were stimulated to produce HIV-1 particles by treatment with PHA/hIL-2 as described. The percentages of GFP-expressing T-cells were determined by direct fluorescence microscopy (inset micrographs) and the supernatants were collected and extracellular HIV-1 particles were concentrated by ultracentrifugation at 44,000 × g at 4°C for 24 h. Relative amounts of extracellular HIV-1 p24Gag protein were determined by immunoblotting. Uninfected HuT-78 lymphocytes were included as a negative control. Relative percentages of extracellular HIV-1 p24Gag protein produced by transfected HIV-1-infected H9HIV-1IIIB lymphocytes are expressed as: 100 − (ΔHIV-1 p24Gag (% GFP-positive cells) and are represented by black bars. The cell pellets were lysed by repeated freeze-thawing and relative luciferase activities were measured using equivalent amounts of total cellular proteins.

To more quantitatively determine the effects of the helicase-minus WRNK577M mutant upon HIV-1 replication, HIV-1-infected H9HIV-1IIIB and uninfected HuT-78 lymphoblastoid cells were co-transfected with pcDNA3.1-GFP and HIV-1 LTR-luciferase in the presence of increasing amounts of CMV-WRNK577M or a C/S vector control. The transfected cultures were stimulated with PHA/hIL-2 to induce high level HIV-1 replication as described. The percentages of GFP-expressing transfected cells were determined by direct fluorescence
microscopy and counting multiple fields (see inset micrographs, Fig. 3C). The cells were harvested by centrifugation and supernatants were layered upon a 70% sucrose, Tris-HCl, pH 7.4, cushion and extracellular HIV-1 particles were concentrated by ultracentrifugation. The relative percentages of HIV-1 p24\textsuperscript{Gag}/GFP-positive cells were determined by immunoblotting and normalization for transfection efficiencies (Fig. 3C). Relative HIV-1 LTR-luciferase transactivation levels were measured to correlate effects of \textit{WRNK\textsubscript{K577M}} upon HIV-1 replication with LTR-dependent transactivation (Fig. 3C). Results in Fig. 3C demonstrate that significant extracellular HIV-1 p24\textsuperscript{Gag} production and LTR transactivation were observed in transfected, PHA/hIL-2-stimulated HIV-1-infected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes, whereas transfected PHA/hIL-2-stimulated HuT-78 lymphocytes did not exhibit detectable HIV-1 p24\textsuperscript{Gag} or LTR transactivation. Increasing amounts of CMV-\textit{WRNK\textsubscript{K577M}} inhibited extracellular p24\textsuperscript{Gag} production, HIV-1 replication, and LTR-dependent transactivation in a dose-dependent manner (Fig. 3C). The C\textsubscript{B}S vector did not alter HIV-1 replication or LTR transactivation (Fig. 3C).

The transfection efficiency of lymphoid cells is generally low (approximately 15–40%). Thus, to determine whether the inhibitory effects of \textit{WRNK\textsubscript{K577M}} upon HIV-1 replication were attributable to partial inhibition in numerous transfected HIV-1-infected cells or near complete inhibition in individual transfected cells, we performed immunofluorescence microscopy to visualize p24\textsuperscript{Gag} production in transfected, PHA/hIL-2-stimulated H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes expressing the Myc-tagged \textit{WRNK\textsubscript{K577M}} mutant protein. Slides were immunostained with anti-HIV-1 p24\textsuperscript{Gag} (red) and anti-Myc tag (green) primary antibodies and appropriate fluorescent secondary antibodies. Surprisingly, p24\textsuperscript{Gag} was almost undetectable in transfected PHA/hIL-2-stimulated HIV-1-infected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes expressing \textit{WRNK\textsubscript{K577M}} (Myc-tagged), whereas high levels of HIV-1 p24\textsuperscript{Gag} were observed in surrounding untransfected cells (Fig. 4A, top panels). HIV-1 replication and p24\textsuperscript{Gag} production are also shown in unstimulated and PHA/hIL-2-stimulated HIV-1-infected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes that were transfected with \textit{C\textsubscript{B}S} vector control (Fig. 4A, lower panels). Unstimulated HIV-1-infected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes chronically produce low levels of virus particles as shown in Fig. 4A (lower right panels). As we had observed that overexpression of wild type WRN stimulates HIV-1 replication (Fig. 3B), we examined whether ectopic WRN (Myc-tagged) influences basal HIV-1 replication in unstimulated transfected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes as determined by immunofluorescence microscopy.

Results in Fig. 4B demonstrate that overexpression of wild type WRN (Myc) induces high level HIV-1 p24\textsuperscript{Gag} (red) in unstimulated transfected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes, compared with untransfected surrounding cells. 4',6-Diamidino-2-phenylindole nuclear staining was included to visualize the entire cell population (Fig. 4B). No significant apoptosis or nuclear condensation was observed in HIV-1-infected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes expressing the \textit{WRNK\textsubscript{K577M}} mutant, however, inhibition of endogenous WRN functions by \textit{WRNK\textsubscript{K577M}} differentially affected (activated/repressed) the transcription of numerous cellular genes in Affymetrix microarray analyses (data not shown).

To better correlate the effects of the dominant-negative \textit{WRNK\textsubscript{K577M}} mutant protein upon HIV-1 LTR trans-activation and virus replication, we transfected H\textsubscript{9\textsubscript{HIV-1IIIB}} lymphocytes with pCDNA3.1-GFP and increasing amounts of CMV-\textit{WRNK\textsubscript{K577M}} or the empty \textit{C\textsubscript{B}S} vector control. Green fluorescent protein expression was visualized by direct fluorescence microscopy and GFP positive-transfected cells were isolated by fluorescence-activated cell sorting (Fig. 5A). We observed that the percentage of GFP positive-transfected cells in total population was ~18–20% based on FACS analyses. The isolated cells were then subcultured in the presence of hIL-2/PHA to induce high level HIV-1 replication. Expression of the \textit{WRNK\textsubscript{K577M}} mutant protein significantly inhibited HIV-1 replication as determined by HIV-1 p24\textsuperscript{Gag} enzyme-linked immunosorbent assays (Fig. 5B). Supernatants from cultured FACSIDFSA-sorted GFP-positive cells were used to infect target HuT-78 lymphocytes, containing the pCV745-HIV-1 LTR-luciferase reporter plasmid, and relative LTR transcriptional activities were measured (Fig. 5C). Samples were normalized using the \textit{tk-Renilla} luciferase (Fig. 5C). These results demonstrate that the inhibitory effects of the \textit{WRNK\textsubscript{K577M}} mutant upon
HIV-1 replication correlate with inhibition of LTR transactivation in transfected H9HIV-1IIIB lymphocytes. We next examined recruitment of the WRN helicase to HIV-1 Tat/LTR complexes in hIL-2/PHA-stimulated HIV-1-infected H9HIV-1IIIB lymphocytes by ChIP analyses. Results in Fig. 5D demonstrate that HIV-1 Tat-specific oligonucleotide PCR primers (40) amplified precipitated products in anti-Tat and anti-WRN ChIPs. No amplification was observed for the nonspecific IgG control ChIP (Fig. 5D). Our findings demonstrate that WRN is an essential cofactor for HIV-1 LTR transactivation and retroviral replication. Immortalized human WRN−/− WS fibroblasts (41) are impaired for HIV-1 LTR transactivation and exhibit reduced basal transcription levels compared with normalized cells containing endogenous WRN. The Tat protein cooperates with WRN on the HIV-1 LTR; and Tat colocalizes with WRN in HIV-1-infected cells/tissues derived from NeuroAIDS patients with primary HIV-1 encephalopathies. We have further demonstrated that the WRN helicase interacts with purifed recombinant GST HIV-1 Tat protein (20) in biochemical GST pull-down experiments. Results from LTR-ChIP analyses, performed on HeLa cells transfected with an episomal HIV-1 LTR-luc construct and a dominant-negative helicase-minus WRN<sub>K577M</sub> mutant interferes with HIV-1 replication.

**DISCUSSION**

The HIV-1 Tat protein transactivates the retroviral LTR through concerted interactions with cellular cofactors, including p300/CBP, PCAF/hGCN5, P-TEFb, SET7/SET9 methyltransferases, SIRT1, Brm SWI/SNF, and SKIP (18–37, 40). The PCAF acetyltransferase has been shown to acetylate Tat on Lys<sup>50</sup>, which stimulates the recruitment of P-TEFb to Tat/TAR-RNA complexes associated with RNA pol II COOH-terminal domain phosphorylation and increases the synthesis of full-length HIV-1 mRNAs (20, 30). Acetylation of Lys<sup>50</sup>/Lys<sup>51</sup> of Tat by p300/CBP and/or PCAF/hGCN5 dissociates Tat/TAR-RNA complexes (20, 35, 39). Mujtaba et al. (29) have demonstrated that Lys<sup>50</sup>-acylated Tat binds to the bromodomain of PCAF that could indirectly tether the retroviral transactivator to the LTR to enhance transcriptional elongation. Tréand et al. (36) have also demonstrated that Lys<sup>50</sup> acetylation interferes with Tat binding to the Brm component of the SWI/SNF chromatin-remodeling complex on the HIV-1 LTR. Intriguingly, Pagans et al. (35) have shown that the histone deacetylase, SIRT1, specifically deacetylates Lys<sup>50</sup> of Tat and may facilitate “recycling” of the transactivator to promote multiple rounds of LTR transactivation.

Our results demonstrate that the WRN helicase is a novel cellular cofactor for HIV-1 LTR transactivation and retroviral replication. Immortalized human WRN<sup>−/−</sup> WS fibroblasts (41) are impaired for HIV-1 LTR transactivation and exhibit reduced basal transcription levels compared with normalized cells containing endogenous WRN. The Tat protein cooperates with WRN on the HIV-1 LTR; and Tat colocalizes with WRN in HIV-1-infected cells/tissues derived from NeuroAIDS patients with primary HIV-1 encephalopathies. We have further demonstrated that the WRN helicase interacts with purified recombinant GST HIV-1 Tat protein (20) in biochemical GST pull-down experiments. Results from LTR-ChIP analyses, performed on HeLa cells transfected with an episomal HIV-1 LTR-luc construct and a dominant-negative helicase-minus WRN<sub>K577M</sub> mutant (41, 43) or using immortalized...
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WRN −/− WS fibroblasts (41), suggest that WRN is required for the stable recruitment of PCAF and P-TEFb to Tat/TAR-RNA transcription complexes on the HIV-1 LTR. These findings are consistent with the HIV-1 LTR transcriptional defect observed in immortalized WRN −/−/WS fibroblasts as well as with inhibition of HIV-1 p24Gag production and retroviral replication by the dominant-negative WRN S577T mutant in transfected, HIV-1-infected H9T4L/H11B lymphocytes. Collectively, our results indicate that the WRN helicase is a novel cellular cofactor essential for HIV-1 transcription and replication.

Mutations of wrn cause Werner syndrome associated with premature aging and cellular senescence (50), which resembles certain symptoms observed during AIDS-related wasting disease, such as loss of skeletal muscle mass, weakness and fatigue, and failure to thrive. Indeed, it is intriguing to speculate that interference with normal WRN functions, as a result of HIV-1 infection and/or Tat binding, could, in part, contribute to an aging phenotype and immune suppression in HIV-1-infected AIDS patients. These studies may help to better understand the molecular events underlying HIV-1 pathogenesis and suggest that WRN might be a plausible new target for anti-retroviral therapy. We have further provided the first evidence that the WRN DNA helicase participates in the recruitment of PCAF/P-TEFb-containing transcription complexes that, mechanistically, may account for the general RNA pol II-dependent transcriptional impairment in immortalized human WRN −/− WS fibroblasts reported by Balajee et al. (1).

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REFERENCES

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