WRNIP1 Controls the Amount of PrimPol

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Werner helicase-interacting protein 1 (WRNIP1) was originally identified as a protein that interacts with WRN, the product of the gene responsible for Werner syndrome. Our previous studies suggested that WRNIP1 is implicated in translesion synthesis (TLS), a process in which specialized TLS polymerases replace replicative DNA polymerase and take over DNA synthesis on damaged templates. We proposed that a novel error-free pathway involving DNA polymerase δ and primase-polymerase (PrimPol) functions to synthesize DNA on UV-damaged DNA templates in the absence of WRNIP1 and the TLS polymerase Polη. Hence, in the current study, we analyzed the relationship between WRNIP1 and PrimPol. We found that WRNIP1 and PrimPol form a complex in cells. PrimPol protein expression was reduced in cells overexpressing WRNIP1, but was increased in WRNIP1-depleted cells. The WRNIP1-mediated reduction in the amount of PrimPol was suppressed by treatment of the cells with proteasome inhibitors, suggesting that WRNIP1 is involved in the degradation of PrimPol via the proteasome.

Key words Werner helicase-interacting protein 1; primase-polymerase; translesion synthesis

INTRODUCTION

DNA replication is constantly threatened by DNA damage or unusual DNA structures. Obstacles such as these, which DNA polymerases cannot overcome, are capable of stalling replication fork progression and are the leading cause of genomic instability and pathogenesis. An important strategy to prevent replication fork stalling is translesion synthesis (TLS), in which specialized DNA polymerases replicate across the obstructing DNA sequences in place of replicative DNA polymerases. The TLS process is conserved from bacteria to humans and involves a large family of proteins known as TLS DNA polymerases. Replicative DNA polymerase stalled at DNA lesions is displaced by an appropriate TLS DNA polymerase, which is subsequently replaced by replicative DNA polymerase after bypassing DNA lesions.

Werner helicase-interacting protein 1 (WRNIP1) interacts with Werner syndrome ATP-dependent helicase, the product of the gene responsible for Werner syndrome, a genetic disease characterized by premature aging. WRNIP1 belongs to an evolutionarily conserved family of proteins that includes Mgs1 in yeast and MgsA/RarA in bacteria. It is also a member of the AAA+ ATPase family of proteins and possesses an ATPase domain in its middle region and a ubiquitin-binding zinc-finger (UBZ) domain similar to the E3 ubiquitin ligase RAD18 in its N-terminal region. RAD18 plays key roles in TLS and mono-ubiquitinates proliferating cell nuclear antigen (PCNA) in response to DNA damage. Ubiquitination of PCNA facilitates the dissociation of replicative DNA polymerase δ (Polδ) and subsequent association of TLS polymerases.

One of the TLS polymerases, Polη, forms a complex with RAD18, promotes mono-ubiquitination of PCNA by RAD18 and interacts with WRNIP1 in cells. In our previous study, we found that WRNIP1 interacts with the DNA-bound form of RAD18 and disrupts the complex. In addition, WRNIP1 interacts with Polδ in vitro and stimulates its activity by increasing the frequency of initiation of DNA synthesis on template primers, in other words, recycling of Polδ. Taken together, these findings suggest that WRNIP1 is involved in polymerase switching in the TLS process.

Previously, we analyzed the functional interaction between WRNIP1 and Polη using chicken DT40 cells and found that the UV sensitivity of Polη−/− cells is suppressed by disruption of the WRNIP1 gene. In fact, in our experiments, the UV-induced mutation frequency of Polη−/− cells was almost abolished in the absence of WRNIP1, suggesting that mutagenic TLS polymerases are not involved in the process used to bypass UV lesions in the absence of WRNIP1 and Polη. These data led us to propose the existence of an alternative approach to error-free bypassing of UV lesions in cells lacking WRNIP1 and Polη. If WRNIP1 participates in polymerase switching by removing Polδ to promote the recruitment of Polη, Polδ will be retained at UV lesions in the absence of WRNIP1 and Polη. Although it is generally assumed that the replicative polymerase Polδ is unable to bypass DNA lesions, it does appear to be capable of bypassing some lesions. Moreover, Polδ is thought to be involved in TLS in vivo. In addition, it has recently emerged that primase-polymerase (PrimPol) is able to restart DNA synthesis at stalled DNA replication forks. Taking our findings regarding WRNIP1 and the reports concerning Polδ and PrimPol into account, we propose the existence of a new error-free pathway in which Polδ and PrimPol co-operate to bypass UV-damaged DNA templates in the absence of WRNIP1 and Polη (Fig. 1A). It is possible that WRNIP1 prevents the association of Polδ and PrimPol with UV-damaged sites and inhibits their function in...
In the current study, we found that WRNIP1 and PrimPol form a complex in cells. In addition, we noticed that the amount of PrimPol protein in cells negatively correlates with that of WRNIP1. This fact does not contradict the proposed function of WRNIP1 described above.

MATERIALS AND METHODS

Cell Culture and Reagents Human 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and were maintained at 37°C in an atmosphere containing 5% CO₂. For overexpression, 293 cells were transfected with constructs encoding tagged human WRNIP1 and PrimPol using Lipofectamine 2000 reagent (Invitrogen Thermo Fisher Scientific, U.S.A.), according to the manufacturer’s instructions. Experiments were performed at 48 h post-transfection. The synthetic small interfering RNAs (siRNAs) (siRNA1: #1164126 and siRNA2: HSS183590) were purchased from Bioneer Corporation (Korea) and Invitrogen Thermo Fisher Scientific, respectively, and were transfected into cells using Lipofectamine RNAiMAX (Invitrogen Thermo Fisher Scientific). Experiments were performed at 72 h post-transfection.

Construction of Plasmids Generation of the human WRNIP1 cDNA has been described previously.²⁸,²⁹ The human cDNA encoding PrimPol was cloned from 293 cells by RT-PCR (primers: fw ATG AAT AGA AAA TGG GAA GCA AAA CTG AAG C, rv TTA CTC TTG TAA TAC TTC TAT AAT TAG...
TTC). A FLAG tag was added to the C-terminus of PrimPol by PCR. The PrimPol-FLAG construct was inserted into the pIRESnEO3 expression vector (TaKaRa Bio, Japan).

**Immunoprecipitation from Cell Lysates** Immunoprecipitation experiments were performed as described previously. Briefly, 293 cells transfected with the PrimPol and WRNIP1 constructs were resuspended in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail [Roche Diagnostics, Swiss Confederation]). The resulting lysate was clarified by centrifugation and incubated with immunoglobulin G (IgG)-Sepharose (GE Healthcare, U.S.A.) at 4°C for 30 min. After a further centrifugation, the supernatant was incubated with anti-FLAG-agarose beads (Sigma-Aldrich Co., U.S.A.) at 4°C for 1 h. The beads were then washed five times with lysis buffer and resuspended in Laemmli sample buffer containing 1 mM dithiothreitol.

**Protein Analysis** For Western blotting, samples were lysed in Laemmli buffer and separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The primary antibodies used in this study were anti-FLAG (anti-FLAG M2 monoclonal antibody; Sigma-Aldrich), anti-myc (anti-c-myc antibody, 9E10; Sigma-Aldrich), anti-PrimPol (anti-PrimPol FW: ab185306; Abcam, England), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9485; Abcam). The secondary antibodies were HRP-linked anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, U.S.A.). ECL Prime (Amersham GE Healthcare) was used for detection.

**Determination of RNA Stability** Decreases in the amount of BrU-labeled RNA were monitored using the BRIC kit (MBL Life Science, Japan). Cells were cultured in medium containing BrU for 24 h, washed to remove BrU, and then harvested. The immunoprecipitated BrU-labeled RNA was isolated according to the manufacturer’s instructions, and cDNA was synthesized using PrimeScript RT reagent kit (TaKaRa) and subjected to quantitative (q)PCR analysis using the SYBR Premix Ex Taq II (TaKaRa). LightCycler 96 System (Roche Diagnostics) was used. PrimPol mRNA level was normalized against that of β-actin (Primers: PrimPol-fw: TTTGCAAGCTCGTCTCTGCTG, PrimPol-rv: ATCTCTGTGCTTCAGGTCCTCC, β-actin-fw: CCTGCTCACCCAGCACAAT, β-actin-rv: GCCGATCTCAACGGAGTACCT). At least three independent experiments were conducted.

**Immunostaining** Cells were loaded on poly-lysine culture slides (Corning Japan, Japan), fixed in 4% paraformaldehyde phosphate buffer solution (Wako, Japan) at room temperature for 15 min, and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline at room temperature for 15 min. Subsequently, the cells were incubated with the primary anti-WRNIP1 (anti-WHIP G-2; Santa Cruz Biotechnology, U.S.A.) and anti-PrimPol (Abcam) antibodies (1:200) at room temperature for 1 h. The cells were then washed three times with phosphate-buffered saline and incubated with the secondary antibodies, Cy3-conjugated anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Jackson ImmunoResearch, U.S.A.), at room temperature for 1 h. Images were obtained using a microscope (Leica Microsystems, Germany).

**RESULTS**

**Detection of the Association of WRNIP1 with PrimPol** To test the hypothesis that WRNIP1 controls the function of PrimPol, we investigated whether these two proteins physically interact in cells. Myc-tagged WRNIP1 and FLAG-tagged PrimPol were overexpressed in human 293 cells, and FLAG-tagged PrimPol was immunoprecipitated from the extracts. A small amount of WRNIP1 was co-precipitated with PrimPol (Fig. 1B), suggesting that the two proteins do interact in vivo.

**PrimPol Protein Levels, but Not mRNA Levels, Were Reduced in Cells Overexpressing WRNIP1** During the course of the experiments described above, we noticed that the expression level of the FLAG-tagged PrimPol protein was reduced in cells overexpressing myc-tagged WRNIP1. Subsequently, we confirmed that the level of endogenous PrimPol protein was also reduced by overexpression of FLAG-tagged WRNIP1 (Fig. 2A). Next, we used a BrU assay to determine whether the reduction in endogenous PrimPol protein expression was attributable to a concomitant reduction in the level of the PrimPol mRNA. To this end, control cells and cells overexpressing WRNIP1 were cultured in the presence of BrU for 24 h, washed, and then cultured in the absence of BrU for a further 24 h (Fig. 2B (a)). BrU-labeled RNA was isolated from total RNA prepared from the cells at 0 and 24 h after

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(A) Schematic overview of the experiment. Human 293 cells were transfected with WRNIP1-FLAG/pIRESnEO3 or pIRESnEO3 (control), cultured for 24 h, and then cultured for a further 8 h in the presence or absence of the proteasome inhibitors lactacystin or epoxomicin. (B) Western blot analysis of PrimPol protein levels in extracts of the cells described in (A). (C) Quantification of the signal intensities of the PrimPol bands shown in (B). GAPDH was used as a loading control for normalization of the data.
the washout, and the levels of PrimPol mRNA in the samples were analyzed by RT-PCR. As shown in Fig. 2B, PrimPol mRNA levels were similar in control (pIRES) cells and those overexpressing WRNIP1 at both the 0 and 24h timepoints (Fig. 2B (b)), suggesting that synthesis and degradation of the PrimPol mRNA were not affected by overexpression of WRNIP1.

Proteasome Inhibition Recovered the Expression Level of PrimPol in Cells Overexpressing WRNIP1 Next, we investigated whether the reduced PrimPol protein expression in cells overexpressing WRNIP1 was due to proteasomal degradation. Cells were cultured for 24h after transfection with pIRES (control) or a vector expressing WRNIP1, and then cultured in the presence or absence of proteasome inhibitors for a further 8h (Fig. 3A). The amount of PrimPol protein in cells treated with proteasome inhibitors was larger than that in untreated cells (Figs. 3B, 3C), suggesting that the reduction in PrimPol protein levels in cells overexpressing WRNIP1 was attributable to proteasomal degradation.

Depletion of WRNIP1 Using a Specific siRNA Increased the Amount of PrimPol To examine the role of WRNIP1 in reducing the amount of PrimPol protein further, we used specific siRNAs to transiently deplete cells of WRNIP1. Western blotting revealed that the PrimPol protein level was higher in WRNIP1-depleted cells than non-depleted cells (Fig. 4A). Next, we analyzed whether the cellular localization of PrimPol is affected by the deficiency of WRNIP1. As shown in Fig. 4B upper panel, in cells transfected with the negative control siRNA, WRNIP1 localized mainly to the nucleus, while PrimPol was located in the nucleus and cytosol as re-

![Fig. 4. PrimPol Expression Is Increased in WRNIP1-Depleted Cells](image)

(A) (a) Western blot analysis of extracts from cells treated with WRNIP1-specific siRNAs. Human 293 cells were transfected with two different WRNIP1 siRNAs or a control siRNA (-) and harvested 72h later. (b) Quantification of the signal intensities of the WRNIP1 and PrimPol bands shown in (a). GAPDH was used as a loading control for normalization of the data. (B) Immunofluorescence analysis of PrimPol in WRNIP1-depleted cells. Human 293 cells were transfected with WRNIP1 siRNA #1, cultured for 72h, and then fixed and stained with a monoclonal anti-WRNIP1 antibody or a polyclonal anti-PrimPol antibody. The secondary antibodies were FITC-conjugated anti-rabbit IgG and Cy5-conjugated anti-mouse IgG. (C) The negative correlation between WRNIP1 and PrimPol protein levels in cells. (D) Hypothetical model of lesion skipping in the absence of WRNIP1 and Polδ.
ported. In WRNIP1-depleted cells, the PrimPol signal was notably enhanced in both the nucleus and cytosol, whereas WRNIP1 was scarcely detected, as expected from the data shown in Fig. 4A.

DISCUSSION

In the current study, we found that WRNIP1 and PrimPol form a complex in cells (Fig. 1B). Unexpectedly, the levels of exogenous and endogenous PrimPol were lower in cells overexpressing WRNIP1 than in control cells (Figs. 1B, 2A). On the other hand, depletion of WRNIP1 led to an increase in the amount of PrimPol protein in cells (Figs. 4A, B). The observed reduction in the amount of PrimPol in cells overexpressing WRNIP1 was caused by protein degradation via the proteasome (Fig. 3), and was not due to mRNA degradation or suppression of mRNA production (Fig. 2B). Thus, it seems likely that WRNIP1 controls the level of PrimPol protein in vivo (Fig. 4C). Negative regulation of PrimPol protein expression by WRNIP1 is consistent with our hypothesis that Polδ and PrimPol function to bypass UV-damaged DNA templates in the absence of WRNIP1 and Polη. We suggest that, in WRNIP1-depleted cells, increased levels of PrimPol are able to access DNA lesions without obstruction by WRNIP1 (Fig. 4D).

WRNIP1 is localized at the replication fork in the presence of DNA damage, and suggested to localize at the replication fork via its association with proteins involved in DNA replication, such as PCNA and Polδ. Two models of the role of WRNIP1 at the replication fork have been proposed. In the first model, WRNIP1 and MgsA/RarA, the Escherichia coli homolog of WRNIP1, prevent collapse of the replication fork. The second model, proposed by us, is that WRNIP1 promotes detachment of Polδ from the stalled replication fork and aids the recruitment of TLS polymerases (Fig. 1A). Similarly, a role of E. coli MgsA/RarA at stalled forks has also been proposed. According to the second model, WRNIP1 may promote the recruitment of Polη at UV lesion-induced stalled replication forks by promoting the exchange of Polδ and decreasing PrimPol levels (Fig. 1A (ii)). In the absence of WRNIP1, even if the exchange of Polδ with Polη is inefficient, Polη rather than PrimPol might function at UV lesion. In the absence of WRNIP1 and Polη, PrimPol levels are increased and Polδ exchange is abolished; hence UV-induced DNA lesions can be bypassed by Polδ and PrimPol, as suggested by us (Figs. 1A (c), 4C).

Interestingly, WRNIP1 was identified as one of p97 binding proteins by proteomic analysis. Both WRNIP1 and p97, also known as valosin-containing protein (VCP), contain motifs shared by members of the AAA+ ATPase family of proteins. AAA+ ATPases are associated with a variety of cellular activities, such as protein unfolding prior to degradation and translocation of proteins. An important function of p97/VCP is that it binds to and extracts ubiquitinated proteins from cellular structures or segregates them from binding proteins, and then facilitates their degradation in the proteasome. Like p97/VCP, WRNIP1 also binds to ubiquitin or ubiquitin chains via the UBZ domain in the N-terminal region of the protein, suggesting that WRNIP1 recognizes ubiquitinated proteins via this domain. Indeed, yeast Mgs1 interacts with polyubiquitinated PCNA via its UBZ domain. It is possible that, like p97/VCP, WRNIP1 acts as a chaperone in the metabolism of PrimPol by receiving a ubiquitin-mediated signal, though ubiquitination of PrimPol has not yet been identified. Considering this proposal, and our current finding that the PrimPol expression is reduced in the presence of WRNIP1 via proteasomal degradation, the following scenario may be proposed: WRNIP1 binds to ubiquitinated PrimPol; then WRNIP1 and/or p97/VCP extract ubiquitinated PrimPol from the complex and subject it to degradation in the proteasome. This hypothesis will be addressed in future studies in our laboratory.

Conflict of Interest The authors declare no conflict of interest.

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