Functional Relationships between Rad18 and WRNIP1 in Vertebrate Cells

Akari Yoshimura, Masayuki Seki, Tomoko Hayashi, Yumiko Kusa, Shusuke Tada, Yutaka Ishii, and Takeki Enomoto

*Molecular Cell Biology Laboratory, Graduate School of Pharmaceutical Sciences, Tohoku University; Sendai 980–8578, Japan; b Shujitsu University, School of Pharmacy; Nishigawara, Okayama 703–8516, Japan; and c Tohoku University 21st Century COE program “Comprehensive Research and Education Center for Planning of Drug development and Clinical Evaluation”; Sendai 980–8578, Japan. Received July 13, 2006; accepted August 19, 2006

© 2006 Pharmaceutical Society of Japan

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by premature aging associated with an early onset of age-related diseases, including arteriosclerosis, malignant neoplasms, melituria, and cataracts. The gene responsible for WS encodes WRN, a member of the RecQ family of DNA helicases. The protein possesses DNA helicase and exonuclease activities. Accumulating evidence suggests that WRN has roles relevant to telomere function since it efficiently prevents telomere degradation and consequent genomic instability. Other functions of WRN are not well understood, although additional roles in many aspects of cellular metabolism may be inferred by its physical interaction with proteins involved in DNA replication, repair, and recombination, including DNA polymerase δ.

To obtain further insights into the function of WRN, we searched for WRN-interacting proteins by a two-hybrid strategy and found a novel protein which we initially denoted WHIP (Werner helicase interacting protein) but which is now called WRNIP1 according to the nomenclatural conventions of HUGO. Interaction between the two proteins was further confirmed by their co-immunoprecipitation from cell extracts.

The amino acid sequence of WRNIP1 is similar to that of replication factor C (RFC), and it also contains the Walker A and B motifs for ATP binding and/or ATPase activity. A homologue of WRNIP1, MGS1, has been identified in budding yeast. Previous studies have shown that overproduction of Mgs1 is lethal in mutants defective in proteins related to DNA replication, such as DNA polymerase δ, RFC, PCNA, and RPA. Moreover, we showed that mutation of mgs1 partially alleviates the growth defect of the pol31 mutant, which bears a mutation in the second subunit of DNA polymerase δ. Consequently, we proposed that Mgs1 (yWRNIP1) interacts with the DNA synthesis machinery to modulate the function of DNA polymerase δ during replication or replication-associated repair. Indeed, we demonstrated that human WRNIP1 interacts with three of the four subunits of human DNA polymerase δ and stimulates its activity.

To investigate the function of WRNIP1 in higher eukaryotic cells, we previously generated WRNIP1 gene knockout cells from chicken DT40 cells. Because the WRNIP1 gene resides on chromosome 2, which is trisomic in DT40 cells, all three alleles were disrupted by gene targeting. WRNIP1−/−/− cells showed a slight elevation of sister chromatid exchange (SCE) and moderate sensitivity to the anticancer drug camptothecin (CPT), an inhibitor of DNA topoisomerase I (Top1), which forms a complex with DNA.

It has been reported that the mgs1 (wrnip1) rad18 double mutation confers synthetic lethality in the budding yeast Saccharomyces cerevisiae. The yeast Rad18 protein is known to function in post-replication repair pathways, including an error-free damage bypass pathway involving Rad30 (Polη) and an error-prone damage bypass pathway involving Rev3/7 (Polζ). The rad18 mutants show hypersensitivity to DNA damaging agents such as ultraviolet (UV), methyl methanesulfonate (MMS), and mytomycin C (MMC). In the case of higher eukaryotic cells, RAD18−/− mouse embryonic stem cells and RAD18−/− DT40 cells also show hypersensitivity to various DNA damaging agents such as UV and MMS. Moreover, both spontaneous and DNA damage-induced sister chromatid exchange (SCE) are elevated in RAD18 knockout vertebrate cells. Interestingly, RAD18−/− DT40 cells were found to be hypersensitive to CPT, while RAD30−/− and REV3−/− cells, which are defective in Polη and Polζ, respectively, are as sensitive to CPT as wild type cells. These data suggest a previously unanticipated role for vertebrate Rad18 in the processing of replication forks that encounter lesions induced by CPT. These circumstances prompted us to examine whether WRNIP1/RAD18 double gene knockout cells exhibit synthetic lethality, as observed for budding yeast, or if they are viable, whether they show a synergistically higher sensitivity to CPT.

Since both DT40 RAD18−/− and WRNIP1−/−/− cell lines have been generated, we constructed WRNIP1−/−/−/−/−...
RAD18−/− DT40 cells by transfecting two RAD18 targeting vectors sequentially into WRNIP−/−−/− cells. We characterized growth, frequency of SCE, and sensitivity to DNA damaging agents including CPT in WRNIP−/−−/−/RAD18−/−−/− cells and obtained unexpected results, as compared to data previously reported for budding yeast. We discuss functional relationships between WRNIP1 and Rad18 in higher eukaryotic cells.

MATERIALS AND METHODS

Cell Culture and DNA Transfection DT40 cells were cultured in RPMI 1640 supplemented with 100 μg/ml kanamycin, 10% fetal bovine serum, and 1% chicken serum at 39.5 °C. For gene targeting, 107 cells were electroporated with 30 μg of linearized RAD18 gene targeting constructs25) using a Gene Pulser apparatus (BioRad, Hercules, CA, U.S.A.) at 550 V and 25 μF Drug-resistant colonies were selected in 96-well plates with medium containing 0.5 μg/ml Puromycin or 20 μg/ml Mycophenolic acid. Gene disruption was confirmed by Southern and Northern blot analyses and RT-PCR.15,25)

Growth Curves Cells (2 × 106) were inoculated and cultured at 39.5 °C for the specified periods. To maintain exponential cell growth, 3 ml cultures were started in 30 mm diameter dishes, from which all cells were successively transferred into 60 and 100 mm dishes. The cells were counted and growth rates were estimated.

Measurements of MMS, CDDP, and CPT Sensitivity Cells (3 × 105) were plated into dishes containing various concentrations of methyl methanesulfonate (MMS), cisplatin (CDDP) or CPT in D-MEM/F-12 medium supplemented with 1.5% (w/v) methicellulose, 1.5% chicken serum, and 15% fetal bovine serum. Visible colonies were counted after 7 to 10 d, and sensitivity to the treated reagent was represented as the percentage of cells forming colonies relative to that of untreated cells.

Measurements of Spontaneous and CPT-Induced SCE Frequencies Cells (5 × 105) were treated with 5 nM CPT for the last 8 h of incubation with 10 μM BrdU for 16 h. The cells were also treated with 0.1 μg/ml colcemid for the final 3 h of incubation to increase the proportion of mitotic cells. The cells were harvested and treated with 75 mM KCl for 20 min at room temperature and then fixed with methanol–acetic acid (3:1) for 30 min. The cell suspension was dropped onto ice-cold wet glass slides and air-dried. The cells on the slides were incubated with 10 μg/ml Hoechst 33258 in phosphate buffer (pH 6.8) for 20 min and rinsed with MacIlvaine solution (164 mM Na,HPO4, 16 mM citric acid, pH 7.0). The cells were exposed to a black light (352 nm) at a distance of 1 cm for 30 min, incubated in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) at 62 °C for 30 min and stained with 3% Giemsa for 10 min.

RESULTS AND DISCUSSION

Generation of WRNIP1−/−−/−RAD18−/−−/− DT40 Cells
The observation that the wrnip1 (mgs1) rad18 double mutant of budding yeast exhibits synthetic lethality27) prompted us to examine whether mutation of the WRNIP1 gene affects higher eukaryotic cells with the RAD18−/−−/− genotype. Since we had RAD18−/− and WRNIP1−/−−/− cells derived from chicken DT40 cells in hand, we generated WRNIP1−/−−/−RAD18−/−−/− DT40 cell lines by sequentially transfecting two RAD18 targeting vectors into WRNIP1−/−−/− cells. Disruption of both RAD18 alleles was confirmed by Southern blotting (data not shown) and by RT-PCR (Fig. 1A). A truncated WRNIP1 mRNA was expressed in WRNIP1−/−−/− and WRNIP1−/−−/−RAD18−−/− cells, and the putative truncated WRNIP1 protein appeared to have no biological activity in vivo, based on our previous characterization.15) Although the wrnip1 (mgs1) rad18 double mutation in budding yeast confers synthetic lethality, WRNIP1−/−−/−RAD18−−/− DT40 cells were viable and grew slightly more slowly than either single mutant (Fig. 1B). We did not detect apparent differences among WRNIP1−/−−/−RAD18−−/−, RAD18−−/−, or WRNIP1−−/−− cells with respect to cell cycle distribution and spontaneous cell death, as monitored by flow cytometry (data not shown).

Additive Increase of Spontaneous SCE in WRNIP1−/−−/−RAD18−−/− Cells
Increases in mitotic recombination frequency are suggested to be the signal of replication defects. Sister chromatid exchange (SCE) (Fig. 2A) in vertebrate cells is thought to be an excellent indicator of such defects.27) The frequency of SCE in WRNIP1−/−−/−RAD18−−/− cells was increased compared to that of either single mutant (Fig. 2B). As reported previously,27) RAD18−−/− cells exhibited an elevation in SCE (Fig. 2B, panel b). Since SCE arise mainly via homologous recombination, the elevation of spontaneous SCE in RAD18−−/−
cells can be explained by the proposal that lesions that remain on replicating DNA in \(Rad18^{-/-}\) cells are channeled to a homologous recombination repair pathway that uses a sister chromatid as a template.\(^{25}\) The frequency of spontaneous SCE in wild-type (a), \(Rad18^{-/-}\) (b), \(WRNIP1^{-/-}\) (c), and \(WRNIP1^{-/-}/Rad18^{-/-}\) (d) cells. The number of SCE in the macro-chromosomes of 100 metaphase cells were counted. Histograms show the number of cells having the indicated number of SCE. The mean and standard error are shown in the upper right corner of each histogram.

Defect in the Induction of SCE by CPT in \(WRNIP1^{-/-}/RAD18^{-/-}\) Cells How does impairment of the \(WRNIP1\) function slightly suppress the severe CPT sensitivity of \(Rad18^{-/-}\) cells? Since overproduction of a protein involved in homologous recombination rescues the (CDDP) or MMS than wild type cells and they show moderate sensitivity to CPT.\(^{15}\) In contrast, \(Rad18^{-/-}\) cells are severely sensitive to all of these drugs, as previously reported (Fig. 3).\(^{15,25,26}\) Here we examined the sensitivity of \(WRNIP1^{-/-}/RAD18^{-/-}\) cells to MMS, CDDP, and CPT. These cells were as sensitive to MMS and CDDP as \(Rad18^{-/-}\) cells, suggesting that \(WRNIP1\) has no role in repairing or tolerating lesions generated by MMS or CDDP, even in the absence of Rad18 (Figs. 3A, B). Surprisingly, the severe CPT sensitivity of \(Rad18^{-/-}\) cells was slightly suppressed by disruption of the \(WRNIP1\) gene (Fig. 3C).

CPT is an inhibitor of DNA topoisomerase I (Top1), which forms a complex with DNA. A Top1-mediated transient DNA single-strand break is formed by reversible transfer of a phosphodiester bond to a tyrosine residue in the catalytic site of the enzyme to form a covalently linked Top1-DNA cleavage complex.\(^{28–32}\) CPT associates with Top1 and stabilizes cleavage complexes, which may result in DNA double-strand breaks (DSBs) followed by the ATR/ATM-dependent phosphorylation of H2AX when DNA replication forks encounter these lesions.\(^{33,34}\) It has been shown that DSBs induced by CPT are mainly repaired by homologous recombination in DT40 cells.\(^{35}\)

**CPT Sensitivity of \(WRNIP1^{-/-}/RAD18^{-/-}\) Cells**

\(WRNIP1^{-/-}\) cells are not more sensitive to cisplatin (CDDP) or MMS than wild type cells and they show moderate sensitivity to CPT.\(^{15}\) In contrast, \(Rad18^{-/-}\) cells are severely sensitive to all of these drugs, as previously reported (Fig. 3).\(^{15,25,26}\) Here we examined the sensitivity of \(WRNIP1^{-/-}/RAD18^{-/-}\) cells to MMS, CDDP, and CPT. These cells were as sensitive to MMS and CDDP as \(Rad18^{-/-}\) cells, suggesting that \(WRNIP1\) has no role in repairing or tolerating lesions generated by MMS or CDDP, even in the absence of Rad18 (Figs. 3A, B). Surprisingly, the severe CPT sensitivity of \(Rad18^{-/-}\) cells was slightly suppressed by disruption of the \(WRNIP1\) gene (Fig. 3C).

CPT is an inhibitor of DNA topoisomerase I (Top1), which forms a complex with DNA. A Top1-mediated transient DNA single-strand break is formed by reversible transfer of a phosphodiester bond to a tyrosine residue in the catalytic site of the enzyme to form a covalently linked Top1-DNA cleavage complex.\(^{28–32}\) CPT associates with Top1 and stabilizes cleavage complexes, which may result in DNA double-strand breaks (DSBs) followed by the ATR/ATM-dependent phosphorylation of H2AX when DNA replication forks encounter these lesions.\(^{33,34}\) It has been shown that DSBs induced by CPT are mainly repaired by homologous recombination in DT40 cells.\(^{35}\)

**Defect in the Induction of SCE by CPT in \(RAD18^{-/-}\) and \(WRNIP1^{-/-}/RAD18^{-/-}\) Cells**

How does impairment of the \(WRNIP1\) function slightly suppress the severe CPT sensitivity of \(Rad18^{-/-}\) cells? Since overproduction of a protein involved in homologous recombination rescues the
lethality of \textit{wrn1} \textit{(mgs1)} \textit{rad18} cells in budding yeast.\textsuperscript{12} we examined the induction of SCE in various cells upon exposure to CPT.

In the presence of CPT, SCE frequencies in wild-type cells and \textit{WRN1} \textit{+/-} cells were higher than those of spontaneous SCE by about 5-fold and 3-fold, respectively, while only 1.3-fold increase in SCE frequency was observed in \textit{RAD18} \textit{+/-} cells. In the case of \textit{WRN1} \textit{+/-} \textit{RAD18} \textit{+/-} cells, no induction of SCE by CPT occurred although the frequencies of spontaneous and CPT-induced SCE in the cells were higher than those of either \textit{RAD18} \textit{+/-} or \textit{WRN1} \textit{+/-} cells (Fig. 4). These data indicate that the induction of SCE by CPT is as proficient in \textit{WRN1} \textit{+/-} cells like as in wild type cells but that it is defective in both \textit{RAD18} \textit{+/-} and \textit{WRN1} \textit{+/-} \textit{RAD18} \textit{+/-} cells. Thus, it is unlikely that the partial suppression of the CPT sensitivity of \textit{RAD18} \textit{+/-} cells by disruption of the \textit{WRN1} gene is due to the induction of recombination.

In conclusion, \textit{WRN1} and \textit{Rad18} in higher eukaryotic cells deal with DNA lesions encountered by replication forks in different pathways, and the lesions that persist in the absence of these proteins are processed by homologous recombination. Thus, both \textit{WRN1} and \textit{Rad18} suppress spontaneously occurring SCE. However, when replication forks encounter lesions induced by CPT, \textit{Rad18} processes lesions in a pathway that results in homologous recombination. The behavior of \textit{Rad18} is not surprising because Sgs1, the yeast homolog of RecQ, suppresses spontaneous homologous recombination, and it is required for homologous recombination induced by DNA damaging agents such as MMS.\textsuperscript{36}\textsuperscript{,} In addition, a pathway involving both \textit{WRN1} and \textit{Rad18}, in which \textit{WRN1} functions upstream of \textit{Rad18}, operates in the presence of CPT. In the absence of \textit{WRN1}, the lesions processed by this pathway are channeled to other pathways, thereby partially suppressing the CPT sensitivity of \textit{WRN1} \textit{+/-} \textit{RAD18} \textit{+/-} cells as compared with \textit{RAD18} \textit{+/-} cells. The elucidation of this pathway is one of our future goals. Recently, it has been reported that yeast Mgs1 (\textit{yWRN1}) interacts with PCNA, which is essential for polymerase \( \delta \) to replicate DNA processively.\textsuperscript{37} Interestingly, both human \textit{WRN1} and yeast Mgs1 interact with DNA polymerase \( \delta \), seem to operate to deal with CPT-induced DNA lesions. Thus, simultaneous deletion of \textit{WRN1} and \textit{Rad18} affects such interplays and then activates other pathway to deal with CPT lesions. Finally, the functional interaction of \textit{Rad18} and \textit{WRN1} is dynamic, and it changes depending on the type of lesion and situation of cells.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES