Proteins and mechanisms involved in modulation of DNA replication and replication fork restart in eukaryotic cells(真核細胞におけるDNA複製と複製フォーク再生機構に関与するタンパク質の解析)

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学位論文題目

Proteins and mechanisms involved in modulation of DNA replication and replication fork restart in eukaryotic cells　（真核細胞におけるDNA複製と複製フォーク再生機構に関与するタンパク質の解析）

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論 文 内 容 要 旨

Organisms are constantly faced with environmental and endogenous DNA-damaging agents, which can cause replication forks to stall. Stalled replication forks generate single-strand DNA (ssDNA) gaps or double-strand DNA breaks (DSBs), which can be lethal lesions or cause genomic instability. Thus, restoring of stalled replication forks is an extremely important process that must be carefully monitored in order to complete replication and to maintain genome integrity. WRN is a RecQ helicase, mutations in which lead to premature ageing and genomic instability, and which has been implicated in various processes of repair and restoring of broken or stalled replication forks. Potentially reflective of its role in replication, WRN has been shown to physically interact with many proteins with functions in DNA replication, examples of which include RPA, PCNA, and DNA polymerase δ (Pol δ). In addition, WRN is covalently modified with SUMO, and interacts with WHIP/WRNIP1, a protein related to RFC subunits. This study has attempted to define the cellular pathways in which WRN and its interacting proteins, WRNIP1, Pol δ, and UBC9/SUMO are involved and their contribution to genomic stability. In addition, by using genetical analysis in *S. cerevisiae*, this study has attmpted to understand how replication fork restart processes take place in eukaryotic cells and how these processes are modulated. The main achievements of this research are summarized below.

1) Mgs1 is involved in maintaining genomic stability and interacts functionally with DNA polymerase δ. The mammalian homologue of Mgs1, WRNIP1/WHIP, was identified as a protin that interacts physically with WRN. *S. serevisiae* cells that lack the RecQ homologue Sgs1 are characterized by a short life span and increased genomic instability. Deletion of *MGS1* in *sgs1* Δ strains dramatically reduces the life span of yeast cells, and *mgs1* Δ *sgs1* Δ cells are characterized by poor viability, slow growth, increased rates of terminal G2/M arrest, and elevated rates of mitotic recombination. Both *MGS1* deletion and overexpression lead to increased recombination frequencies, and Mgs1 overproduction causes lethality or growth defects in mutants of the Pol δ complex, such as *rfc, pcna,* and *pol δ* mutants. In the absence of Mgs1, PCNA has reduced ability to suppress the replication defect of *rfc5-1* mutants at high temperatures, suggesting that Mgs1 is actively involved in replication or restoration of stalled replication forks. *MGS1* deletion suppresses the temperature sensitivity, hydroxyurea (HU) and methyl methanesulfonate (MMS) sensitivity of *pol δ* mutants, as well as the synthetic lethality caused by simultaneous mutation in two different subunits of Pol δ. The results of this study indicate that Mgs1 interacts with the DNA polymerase δ complex and modulates its function during replication restart by facilitating Pol δ-mediated template switch repair synthesis.
2) Red18 and other proteins involved in the error-free damage bypass postreplication repair (PRR) interact functionally with DNA polymerase δ during replication restart. This study has found that mutations in *RAD18* and *MMS2* suppress the temperature sensitivity of *hys2-1* mutants, which are strains carrying mutations in the gene coding for the second subunit of Pol δ, Pol31. *rad18* Δ suppresses also the temperature

sensitivity of *pol3-1* strains, which carry mutations in the catalytic subunit of Pol δ, as well as the synthetic lethality of *pol3-1 pol32 Δ* cells. In addition, *hys2-1* and *pol3-1* partly suppress the hypersensitivity of *rad18 Δ* stains towards UV irradiation or MMS damage. These results suggest that Red18 and Pol δ functionally interact during replication and replication bypass processes and reciprocally modulate their functions.

3) **Delineation of the pathways employed to restart replication and the role for Pol δ during replication restart.** Accumulating knowledge from bacteria and lower eukaryotes indicates that two pathways, the damage tolerance pathway and the recombinational repair pathway, are pivotal in supporting replication when DNA damage is present. The general view of the process is that when replication polymerases stall at replication blocking sites, replication can continue by bypassing the lesions with specialized TLS polymerases, or by transient template switch to the undamaged sister chromatid (error-free PRR). Otherwise, the stalling can lead to DSBs, which are repaired by recombinational repair . The first two mechanisms are known as the damage tolerance pathway controlled by *RAD18*, while the last one is known as the homologous recombinational repair pathway (HR). In addition, there appears to be an alternate damage avoidance (ADA) controlled by *MGS1*, inactivation of which is lethal in combination with mutations in genes involved in the error-free branch of PRR such as *RAD18* and *RAD6*. This study has found that both ADA and error-free PRR pathways promote replication restart by facilitating Pol δ -mediated repair synthesis. Mutations in genes of the error-free PRR or ADA pathways do not lead to growth defects when combinad with mutations in the recombination repair pathway, but *pol δ* mutant cells show severe growth defects and synergistic HU, MMS sensitivities when combined with mutations in the HR pathway. This result suggests that when stalled replication forks cannot be restored by Pol δ -mediated repair synthesis, cells rely on HR to activate replication. Consistent with this model, this study has found that *mgs1 Δ* and *rad18 Δ* mutations suppress the replication defects of *pol δ* strains, but this suppression requires an active HR. In addition, when the *RAD18* pathway is inactive, mutations in Pol δ can rescue the high sensitivity of cells in response to DNA damaging agents, but again cells become dependent on HR for survival. The ADA and error-free PRR damage avoidance pathways appear to be the primary option in reactivating stalled forks since their simultaneous inactivation leads to cell death. However, they both contribute to fork restart by modulating Pol δ function, and in conditions that make the Pol δ complex unavailable or unstable, cells must make use of the HR pathway for repairing or bypassing replicative blocks.

4) **Rad18/Rad5/Mms2-mediated multi-ubiquitination of PCNA is essential for its ability to promote replication restart.** Protein ubiquitination functions as a signal for various processes, including protein degradation and localization, DNA repair and chromatin structure. PCNA, a processivity factor with essential functions in replication and repair, is a ubiquitination substrate. This study has found that Rad18 interacts functionally with Pol δ and promotes Pol δ -mediated replication restart of stalled forks. Since Rad18 mediates ubiquitination of PCNA and potentially of other replication proteins, this study has examined the effect of PCNA ubiquitination on replication. The results of this study suggest that the Rad18/Rad5/Mms2-dependent

multiubiquitination of PCNA is essential for replication completion perhaps through a copy-choice type of DNA repair synthesis. In addition, the results of this study established the copy-choice type of DNA repair synthesis not merely as a damage avoidance pathway that functions to bypass DNA lesions induced by DNA damage, but also as a major pathway of restarting replication forks.

5) UBC9/SUMO pathway is implicated in DNA replication and repair. WRN helicase was found to be covalently modifird with SUMO, and interact with Ubc9. This study has found that Sgs1 and other proteins implicated in DNA replication such as Mgs1, Top3, PCNA, and Pol δ subunits interact with SUMO in the yeast two-hybrid system and are thus potential SUMO targets. By analyzing the phenotypes of *ubc9-1* mutants in which sumoylation of targets is known to be impaired, this study has found that *ubc9-1* mutants are hypersensitive to agents that induce S-phase arrest or cause DNA damage, and activate the checkpoint protein Rad53 at semi and non-permissive temperatures. At high temperatures, *ubc9-1* cells undergo G2/M cell cycle arrest, in a manner that is partly dependent on functional damage checkpoint proteins. In addition, this study has found that *ubc9-1* cells require the functions of *SGS1/MUS81* to recover from replication stress. Genetical epistasis tests performed in this study have indicated that cells rely on recombination repair to restart replication in the absence of a functional *UBC9*, and that sumoylation of targets might affect the ability of cells to undergo error-free post-replication repair. *ubc9-1* mutation suppressed the MMS and UV sensitivities of *rad5* Δ mutants, but increased the MMS sensitivity of *mms2* Δ mutants, which are known to be deficient strictly in error-free PRR and multi-ubiquitination of targets. In addition, the temperature sensitivity of *ubc9-1* cells at semi-permissive temperatures was suppressed by *rad5* Δ and *mms2* Δ mutations. The results suggest that the UBC9/SUMO pathway is implicated in DNA replication and repair, and that sumoylation and ubiquitination are inter-related and might have regulatory roles on each other.

6) Roles of damage checkpoint in activating DNA translesion synthesis replication bypass. When cells attempt to replicate damaged DNA, the replication checkpoints and the intra-S DNA damage checkpoints are activated. Checkpoint activation triggers cell cycle arrest and DNA repair, but the mechanisms underlying these processes remain yet to be elucidated . The results of this study suggest a link between damage checkpoints and translesion synthesis (TLS) replication bypass. In response to UV and MMS induced lesions, Rad24 and Rad17 are required to induce mutagenesis. It is known that mutagenesis occurs during replication, and indeed loss of *RAD24* dramatically reduced the ability of *pol* δ mutants to cope with fork collapse events such as those induced by high temperature, DNA damage, or depletion of nucleotide pools. As a response to replication fork stalls, recombinational repair, and PRR pathways are activated. Loss of Rad24 increases the HU sensitivity in *rad51* Δ and *rad52* Δ mutants, and almost rends cells incapable of dealing with HU induced fork collapse in the absence of *RAD5*. This result indicates that Rad24 and Rad5 pathways are absolutely required to promote fork restart in response to nucleotide depletion. Mutations in Pol ζ or Pol η genes do not significantly increase the HU sensitivity of either *rad24* Δ or *rad5* Δ strains, but *rad24* Δ *pol* ζ Δ strains show a synergitic increase in MMS

and UV sensitivity. Interestingly, spontaneous mutagenesis in recombination and PRR mutants requires *RAD24* function, suggesting that Rad24 might facilitate damage-bypass mutagenesis when error-free replication bypass pathways are not available or when the damaged connot be removed or bypassed. Although studies in *S. pombe* have shown physical and genetic interactions between the TLS polymerase DinB and damage checkpoints, in *S. cerevisiae*, this study provides the first report of that kind.

In conclusion, the results of this study allowed the delineation of the main pathways involved in replication restart. DNA polymerase δ has a central role in replication restart and its function is modulated by Mgs1 and Rad18. Both ubiquitination and sumoylation are implicated in replication: multiubiquitination of PCNA is pivotal for promoting fork restart through template switching and completion of replication, and sumoylation of tergets regulates the ability of cells to restore forks and undergo PRR. Checkpoint proteins monitor replication and activate repair in response to replication blocks, and in the presence of excess DNA damage they activate TLS. The results of this study help to elucidate the mechanisms that regulate replication fork restart following stalling or collapse of the replication forks.

審査結果の要旨

ウェルナー症候群は早老症として知られる代表的な疾患で，患者由来の細胞ではDNA複製に欠陥があることが知られている。また，その原因遺伝子産物WRNと結合するタンパク質として，DNA複製に必須なRPA，PCNA，FEN1，DNA polymerase δ（Pol δ）が報告されている。本研究は，我々の研究室で，WRNと結合するタンパク質として新たに発見されたWRNIP1（Werner helicase interacting protein 1）の酵母ホモローグの機能の解析を行うとともに，WRNIP1と機能的関連をもつタンパク質の機能をDNA複製と複製フォーク再生機構に焦点を当て解析したものである。

まず始めに，*WRN*の酵母ホモローグである*SGS1*と*WRNIP1*の二重遺伝子破壊株を作製して解析することにより，二重遺伝子破壊株ではDNAの組換え頻度が上昇し，酵母の分裂寿命が短縮することを見出した。また，Wrnip1をDNA複製に関与する酵素・タンパク質をコードする遺伝子に変異をもつ株に過剰発現したり，これらの変異株にさらに*WRNIP1*の変異を導入して解析することにより，Wrnip1はPol δ，PCNA，RFCと機能的関連をもち，Pol δとは直接結合する可能性があることを明らかにした。さらに，Pol δの変異株の変異を抑制する遺伝子のスクリーニングを行い，複製後修復で機能する*RAD18*，*MMS2*を見出した。

次に，Pol δとWrnip1，Sgs1，Rad52，Rad18，Mms2との機能的関連を解析することにより，「DNA複製と複製フォークが崩壊した際，その再生には相同組換え経路とPol δの機能を要求する複製後修復経路が主に働き，両者がお互いに調節しあう」というモデルを提出した。

最後に，タンパク質の修飾から上記の調節機構の解明を目指し，SUMO化，ユビキチン化に注目して解析を行った。その結果，Wrnip1，Pol δ，Sgs1，PCNAがSUMO化されることを示す結果を得た。また，Rad18，Mms2がユビキチン化に関与し，PCNAがユビキチン化されるという報告があったことから，PCNAのユビキチン化，SUMO化を中心に解析を行い，「DNA複製フォークの再生において，PCNAのユビキチン化を介した複製後修復経路の活性化が重要であり，PCNAのSUMO化はユビキチン化と拮抗し，相同組換え経路を活性化する」という可能性を示した。

このように，本研究はWrnip1の機能の解明に大きく貢献しただけでなく，現在真核細胞のDNA 複製に関する研究で注目を集めているDNA複製フォークの再生機構の解明にも大きく貢献するものである。よって，本論文は博士（薬学）の学位論文として合格と認める。