On the role of Rad5 protein during the rescue of stalled replication forks

Thesis of Ph. D

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Introduction and aims

Eukaryotic cells have evolved sophisticated means to recognize and repair damaged DNA. While these mechanisms are remarkably efficient, damaged DNA can often left unrecognized for a long time. Such unrepaired DNA lesions in the template strand can block the progression of the replication fork and arrest cells in S-phase leading to fatal consequences. Discontinuity of replication upon DNA damage was experimentally demonstrated indeed, but the results also revealed a mechanism by which the single-stranded gaps can be sealed during S-phase. This process is referred to as post-replicational repair (PRR). Later, it turned out that damaged bases are persistent after PRR, thus, it is not a repair but rather a bypass (damage tolerance) mechanism in strict sense.

To identify genes responsible for PRR in yeast, various mutant strains were examined for their ability to resolve the single-stranded gaps created after UV irradiation. These studies established that PRR is dependent upon RAD6 and RAD18 genes, whose protein products form a ubiquitin ligase and conjugation enzyme complex. Although the mechanisms associated with the Rad6–Rad18 heterodimer are not fully understood, the requirement of RAD6 and RAD18 genes for damage-induced mutagenesis indicates that mutagenic bypass by Trans-Lesion Synthesis (TLS) polymerases is one of the tolerance mechanisms. However, successful TLS depends on the chemistry of the damaged base and whether the particular polymerase can handle it. Considering the myriads of possible DNA lesions damage bypass by TLS polymerases unlikely to occur in all the cases, and indeed, strains mutant for TLS polymerases show no spectacular defect in PRR. This indicates the existence of a major alternative bypass mechanism.

Upon DNA damage, a considerable portion of DNA is replicated by conservative manner, so that the nascent strand could template DNA synthesis. In order to provide a plausible explanation for this puzzling result and to explain the mechanism of PRR the model of the replication fork reversal was invoked. During this process, the nascent strands anneal to form a template-primer

junction allowing further extension of the blocked leading strand using the newly synthesized lagging strand as template. The resulting four-way junction structure resembles of a chicken-foot, a term widely used for its description. Resetting the fork by branch migration of the “chicken foot” completes bypass of the lesion. Importantly, since the intact nascent strand is used for damage bypass, this process may independent on the chemical nature of the damage. As a consequence, template switching by fork reversal must be an essentially error-free mechanism.

PRR is mainly mediated by the error-free sub-branch of the RAD6/RAD18 pathway, which is composed of the RAD5, MMS2, UBC13 and POL30 genes. Rad5 acts as a ubiquitin ligase for the Ubc13/Mms2 ubiqutin-conjugating enzyme complex. The substrate of this reaction is PCNA, already monoubiquitylated by Rad6/Rad18. How polyubiquitylated PCNA can coordinate PRR is unknown, however, Rad5 mutant for its E3 function is as defective in PRR as the strain carries the complete deletion for this gene.

Rad5 consist of seven conserved helicase-like motifs and belongs to the SWI/SNF2 superfamily of proteins. Members of this family are DNA dependent ATPases and Rad5 indeed possesses such an activity. DNA dependent ATPase activity is also a hallmark of enzymes involved in various DNA metabolic processes such as unwinding double stranded DNA. Fork reversal requires rearrangement of DNA strands around the growing point of the replication fork, thus Rad5 seemed to be a promising candidate for the effector of this process, which supposed to be performed by a helicase. Supporting this notion, Rad5 mutant for its ATPase function shows complete defect in PRR. Moreover, the in vivo role of Rad5 in fork reversal has already been demonstrated. Nevertheless, despite the above-mentioned clues Rad5 does not possess a helicase activity.

The main goal of this thesis is the detailed biochemical analysis of Rad5 in order to understand its role in replication fork reversal. Our central hypothesis was that Rad5 may perform fork reversal without any inherent helicase activity. Based on this idea we formulated the specific aims that are listed below.

I. We have to set up an experimental system powerful enough to distinguish helicase-like processing of a replication fork from its reversal. In order to achieve this goal we compare the activity of Rad5 on heterologous and homologous oligonucleotide based replication fork model substrates. If it is possible to detect any ATP dependent activity of Rad5, we will thoroughly characterize the reaction in order to gain insight into its mechanism.

II. In order to more closely approximate the *in vivo* situation we construct a replication fork, which has kilobases long arms and asymmetry at the base of the fork. Using this experimental system, we further characterize the mechanism of Rad5 by probing the progressive nature of the reaction.

III. In order to understand how Rad5 could perform replication fork reversal its substrate requirement during translocation along DNA will be tested. This set of experiments will decide between the possibilities whether Rad5 is a single-stranded, or a double-stranded DNA translocase.

Results and discussion

The biochemical data presented in the thesis give compelling evidences for a direct role of Rad5 in mediating error-free lesion bypass by replication fork reversal, a conclusion that is in keeping with genetic observations previously made by others. The novel findings of our work are listed below.

I. Using oligonucleotide based replication fork model substrates we showed that Rad5 is unable to unwind a model replication fork structure containing heterologous arms, however, it can process a model replication fork with homologous arms. The outcome of this reaction is fully consistent with the hypothesis that Rad5 has no helicase, but only fork reversal activity. We demonstrated that Rad5 processes a homologous fork in a highly concerted manner using the energy provided by hydrolysis of ATP. We also presented evidence for branch-migrating activity of Rad5

II: We found that Rad5 can process a plasmid sized asymmetric replication fork model substrate in an ATP dependent manner and could generate as long as 863 basepair regressed arm, the size that is comparable to the observed extent *in vivo*. We were able to demonstrate the progressive nature of the reaction. We also demonstrated that Rad5 acts without extensive disassembly of the fork.

III. We demonstrated the double-stranded DNA translocase activity of Rad5 and that the integrity of both strands in the double-stranded context is required for efficient translocation. We presented a model that may faithfully describe some important aspects of the fork reversal process and considers the advantages and disadvantages of the reaction.

Rad5 possesses the biochemical activity that is congruent with the tenets of the fork reversal model and was missed for a very long time. Rad5, as many but not all members of the SWI/SNF2 superfamily, is a double-stranded DNA translocase, which activity provides an important clue for understanding the fine mechanistic details of the for reversal reaction.

We propose that Rad5 can act similar to chromatin remodelling enzymes, but it removes DNA roadblocks during translocation; we use the term “DNA strand remodelling” to circumscribe this activity. This is not a far-fetched term, since chromatin remodelling, fork reversal and branch migrating activities are not ubiquitously shared within SWI/SNF2 family. Clearly, may all these activities stem from the common translocating activity the fine mechanistic differences appear to restrict the act of these enzymes to the appropriate *in vivo* context. Rad5 is just the second example for that SWI/SNF2 family members can perform strand transfer on structured DNA substrates. Our study may help to understand the function of those members that are clearly lack any chromatin remodeling activities.

Rad5 dependent template switching comprises as much as 90% of damage bypass events during replication, and the rate of gross-chromosomal rearrangements increases as much as 200-fold in its absence. This is what can be expected if stalled forks are resolved by non-safety mechanisms, such as at the price of fork collapse.

Increased genomic instability and consequent complex genomic rearrangements are hallmarks of malignantly transformed cells in higher order eukaryotes. The role of Rad5 in maintenance of genomic stability suggests that inactivation of its human counterparts can be a primary event which promotes the accumulation of mutations and rearrangements during formation of a stably transformed malignant clone. Future studies will very likely establish such a tumor-suppressor role for the human homologous of Rad5 and identify their role in the pathogenesis of human diseases.

List of publications:

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(The theses are based on the data presented in Blastyák et al., 2007.)