Summary of PhD thesis

Structure function study of the yeast Rad18 protein

Orsolya Frittmann

Supervisor: Dr Ildikó Unk
principal investigator

Doctoral School of Biological Sciences
University of Szeged
Department of Informatics and Natural Sciences

Lóránd Eötvös Research Network
Biological Research Centre
Institute of Genetics

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Introduction

The genetic information in the cells is exposed to several internal and external damaging factors. During the evolution several mechanisms formed to repair and preserve the genetic information of the DNA. Despite the available repair pathways sometimes damages can't be resolved till the S-phase which leads to stalling of the replication fork, double-stranded DNA breaks and chromosomal breaks. In order to maintain genome integrity a few mechanisms exist which can help the replication machinery bypass the damaged base. These mechanisms together are called DNA damage tolerance (DDT) pathways.

From the several different DDT mechanisms our group focuses on understanding of the Rad6/Rad18-governed DDT pathway. When the replication machinery encounters a damaged base, it stops and the Rad6/Rad18 complex ubiquitinates the PCNA molecule on the Lysine 164. This ubiquitination initiates the change of replicative DNA polymerase to a translesion polymerase with a flexible active centre which enables the continuation of the DNA synthesis through the damaged base. When the Mms2/Ubc13-Rad5 complex polyubiquitinates the already ubiquitinated PCNA molecule the error-free subpathway is activated, which works via fork regression or template switching.

Without the Rad6/Rad18 complex the DDT pathway can't work. Despite the Rad18's essential function it's role and structure are not well described yet. As the result of long years of research in the topic six domains were characterized in the Rad18 protein's sequence. The first few scientific papers described it as an ATPase based on the Walker A type nucleotide binding motif localized in the C-terminal part of the protein. Also, in the C-terminal region we can find a domain which is responsible for the interaction with the ubiquitin conjugase, Rad6. The N-terminal part contains a RING domain which is common in the E3 ubiquitin ligase family and a SIM motif which is necessary for the binding of the SUMO molecule. The Zn-finger and the SAP domain which functions are not clear yet, are located in the middle of the protein. Our goal was to clarify the exact role of the known domains in the Rad18 protein and describe the function of the remaining parts in the DNA damage tolerance pathway.

Goals

Our aim was to clarify the already known domain's function and characterize the uncharacterised parts of the Rad18 protein.

We designed the following steps:

1. Making of deletion and point mutation containing *RAD18* genes.
2. Preparing of mutant Rad18 as single Rad18 expressing source in the yeast strains.
3. From the series of mutants find the ones which shows sensitivity upon DNA damaging agents and can possibly affect Rad18's role in damage tolerance pathway.
4. Using genetic analysis methods describe which subpathway is affected by the given deletion/point mutation.
5. Understand the interaction pattern between the mutant proteins and the well-known interaction partners. Investigate whether the deleted regions are necessary for the formation of Rad18 interactions.
6. Check the mutant Rad18 proteins DNA binding ability.

Applied methods

Gene knockout with site directed mutagenesis

DNA manipulation, cloning

Yeast genetics, measuring the mutagenic rate

Protein-protein interaction

Purifying recombinant proteins

Checking the DNA binding potential

Results and discussion

During this work we investigated 8 deletion and one point mutant Rad18 protein's role in the DNA damage tolerance pathway. When we started our work there was no function connected to the big protein regions in the C- and N- terminal part of the protein so we designed 3-3 deletion constructs to cover these areas. In the meantime, researchers located the SIM (SUMO interacting motif) motif between the RING and the Zn-finger domain which is necessary for the interaction with SUMO molecule. There was no information about the role of the SAP domain so in order to describe the function of this region we designed a mutant which deletes half of the domain and another one which removes 42 amino acids in front of the SAP domain. To describe the role of the part between the Zn-finger and the SAP domain we designed a mutant which lacks 62 amino acids in this region. In most of the cases the C2HC sequence motif of the Zn-finger proteins is conserved. In this case we disrupted the sequence by point mutations which inhibit the formation of the domain's authentic structure.

In order to describe the role of the C-terminal part of the protein we used 3 different sized deletion constructs. We learned that there is no essential function connected to the C-terminus because the deletions didn't affect Rad18's role in the DDT.

Similarly, in order to the investigate the C-terminal part's role we designed 3 different deletion constructs to describe the function of the N-terminal region. We noticed that the protein containing the biggest deletion showed an extreme sensitivity upon DNA damaging agents which means that the missing part has an essential role in the DDT pathway. By using yeast two-hybrid and GST pull-down experiments we checked whether this protein is able to interact with the well-known partners. It showed that Rad6-binding and dimer formation abilities were not affected in this case however the deletion containing protein wasn't able to interact with Rad5. The lack of this interaction doesn't lead to the experienced sensitivity so we thought that interaction between the Rad18 and the PCNA molecule is might be inhibited by the deletion. We couldn't prove this assumption in our experimental setups so the cause of this extremely sensitive phenotype is still an open question.

The other two proteins containing N-terminal deletion showed intermediate sensitivity upon DNA damaging agents which lead us to conclude that the function of Rad18 is restricted in the DDT pathway. With epistasis analysis assays we were able to prove that the missing parts affect the work of the fork regression/template switching subpathway. The yeast two-hybrid experiment showed that the deletions inhibited the interaction between the Rad18 and the Rad5

proteins which interaction is the main regulator of this subpathway. Using these deletion constructs we were able to locate the Rad5-binding sequence to amino acid 155-190 region. It is possible that in the case of the protein containing the biggest deletion 80-115 amino acids has essential role which is necessary for the Rad18's functions, and the lack of this region causes the extremely sensitive phenotype.

Right after our N-terminal deletion series we can find the Zn-finger domain in the Rad18 protein sequence which we disrupted by using point mutations (CC190,193GG). The Zn-finger point mutant showed similar results as the two N-terminal deletion mutants, it resulted intermediate sensitivity upon DNA damaging agents and epistatic relation with the Rad5 pathway. In yeast two-hybrid experiments this protein was able to interact with the well-known partners, except with the Rad5 protein. Upon these findings it seems that the Zn-finger is also necessary for the Rad5-Rad18 interaction which means that the interaction surface is even bigger than we initially thought, the region between amino acids 155-210 is responsible for this binding.

In order to describe the role of the central region of the protein we designed a mutant which lacks the region between the Zn-finger and the SAP domain (M) and a mutant where half of the SAP domain is deleted. Both of them showed similar sensitivity upon DNA damaging agents as the *RAD18* knock-out strain. Based on literature data, SAP domain is responsible for DNA binding. We were able to confirm this function as in our experiments the protein lacking the SAP domain wasn't able to bind the ssDNA, this can lead to the extreme sensitive phenotype. We were curious about the interaction pattern of the SAP mutant protein whether by disruption of the protein structure via deletion was able to inhibit the interactions. In our experiments the SAP mutant protein was able to interact with the well-known partners.

Working with the M mutant showed unexpected results. The deletion protein wasn't able to interact with Rad5 and it couldn't bind the ssDNA. Based on this we concluded that the region between the Zn-finger and the SAP domain acts as a bridge, it helps in the formation of the right 3D structure by keeping the two domains apart.

Results:

1. The C-terminal part of the Rad18 has no role in the DDT pathway.
2. The region between amino acids 155-246, which includes the Zn-finger motif, is responsible for Rad5-binding.
3. The helix-loop-helix motif in the SAP domain is necessary for the ssDNA binding.

4. The region between the Zn-finger and the SAP domain has a role in the Rad5 and ssDNA binding by helping to form the right conformation.

Publications

1. Publications for the doctoral process

Frittmann O, Gali VK, Halmai M, Toth R, Gyorfy Z, Balint E, Unk I
The Zn-finger of Saccharomyces cerevisiae Rad18 and its adjacent region mediate interaction with Rad5.
G3 (Bethesda). 2021 Feb 11. doi: 10.1093/g3journal/jkab041.
PMID: 33570581 IF (2020): 2.781

Vamsi K. Gali, Eva Balint, Nataliia Serbyn, **Orsolya Frittmann**, Francoise Stutz, Ildiko Unk
Translesion synthesis DNA polymerase η exhibits a specific RNA extension activity and a transcription-associated function
Sci Rep. 2017; 7: 13055. Published online 2017 Oct 12. doi: 10.1038/s41598-017-12915-1
PMCID: PMC5638924, IF (2020): 4.12

2. Publications in international scientific journals

Miklos Halmai, **Orsolya Frittmann**, Zoltan Szabo, Andreea Daraba, Vamsi K. Gali, Eva Balint, Ildiko Unk
Mutations at the Subunit Interface of Yeast Proliferating Cell Nuclear Antigen Reveal a Versatile Regulatory Domain
PLoS One. 2016; 11(8): e0161307. Published online 2016 Aug 18. doi: 10.1371/journal.pone.0161307
PMCID: PMC4990258, IF (2020): 2.87

Vamsi K. Gali, Eva Balint, Nataliia Serbyn, **Orsolya Frittmann**, Francoise Stutz, Ildiko Unk
Translesion synthesis DNA polymerase η exhibits a specific RNA extension activity and a transcription-associated function
Sci Rep. 2017; 7: 13055. Published online 2017 Oct 12. doi: 10.1038/s41598-017-12915-1
PMCID: PMC5638924, IF (2020): 4.12

Frittmann O, Gali VK, Halmai M, Toth R, Gyorfy Z, Balint E, Unk I
The Zn-finger of Saccharomyces cerevisiae Rad18 and its adjacent region mediate interaction with Rad5.
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PMID: 33570581 IF (2020): 2.781

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3. Other scientific publications

3.1 Conference presentations:

- Central European Genome Stability and Dynamics Meeting, October 15th-16th, 2016, Zagreb
- XXXII. National Scientific Student Conference, 2015, Pécs
- FIBOK 2014, Young Biotechnologists National Conference 2014, Szeged
- Innovation In Science- Doctoral Student Conference, 2014, Szeged

3.2. Poster presentations:

- 6. Central European Genome Stability and Dynamics Meeting, 2015, Szeged
- Straub Days, 2013, Szeged