The role of human Spartan in the repair of DNA–Protein Crosslinks

Ph.D. thesis summary

Eszter Sági-Zsigmond

Supervisor:
Lajos Haracska Ph.D., D.Sc.
scientific adviser

Biological Research Centre
Institute of Genetics

Doctoral School of Biology
University of Szeged
Faculty of Science and Informatics

Szeged
2021

Introduction

Our DNA is constantly exposed to different exogenous and endogenous factors that may cause DNA damage, which can lead to the stalling of the replication fork. Understanding DNA repair mechanisms, discovering and characterizing the members of these pathways could provide key elements for the prevention of carcinogenesis, mutagenesis, and progeroid syndromes. DNA-protein crosslinks (DPCs) are formed when proteins covalently and irreversibly bind to the DNA. Any protein (histones, transcription factors, DNA repair and replication proteins) that is in the vicinity of the DNA can be covalently bound to the DNA. DPCs can be induced exogenously by different reactive aldehydes, ionizing irradiation, UV light, etc. or endogenously in our cells during metabolism. DNA-protein crosslinks, due their bulky size, inhibit transcription and DNA replication. Although DPCs are one of the most frequent and harmful DNA alterations, because they interfere with replication and transcription, their mechanism of repair and elimination is hardly known. Until recent findings, the repair of DPCs was considered to be performed only by canonical DNA repair pathways such as NER (Nucleic Excision Repair), homologous recombination, and the Fanconi anaemia pathway. A recent discovery in yeast revealed that during replication a newly identified protein, *wss1*, exhibits a DNA-dependent activity in degrading DPCs. The yeast *wss1* protein is suggested to be a human Spartan homologue because both proteins contain a metalloprotease domain: Spartan an SprT domain and Wss1 a WLM domain.

Spartan, a new protein of the DNA Damage Tolerance (DDT) pathway, was discovered and characterized in 2012 by our lab and others. We described

that Spartan promotes genomic stability by regulating the choice of rescue of the stalled replication fork and that the silencing of the protein results in a 2.5-fold increase in sister chromatid exchange. Spartan's essential role in the maintenance of chromosomal and genome integrity is supported by the facts that Spartan mutations were found in three patients from two unrelated families with early onset of hepatocellular carcinoma and progeroid features and complete loss of Spartan leads to early embryonic lethality in mice.

Aims

Our aim was to describe and understand the function of the SprT-like metalloprotease domain of human Spartan and to characterize and highlight the role of human Spartan in the repair of DNA-protein crosslinks.

To answer our questions, we designed experiments aimed at:

- Investigating, describing, and characterizing the DNA-dependent protease activity of human Spartan
- Investigating whether human Spartan has a role in the repair of DNA-protein crosslinks
- Developing and characterizing an S-phase- and DPC-specific BrdU comet assay
- Investigating whether the SprT, DNA-binding, PIP and UBZ domains of Spartan contribute to cells' resistance to DNA-protein crosslinks
- Investigating whether Spartan facilitates immediate bypass of formaldehyde-induced DNA damage
- Investigating whether Spartan and Rad18 act together in protecting the genome from DPCs

Methods

- To create the plasmid constructs necessary for the purification of the human Spartan protein, restriction digestion, LR reaction, ligation, plasmid purification, bacterial and yeast transformation were performed.
- The proteins were purified with GST affinity chromatography and the purification was verified with gel electrophoresis using denaturing polyacrylamide gel.
- For the description of the DNA-dependent protease activity of the human Spartan protein, electrophoretic mobility shift assay using native polyacrylamide gel, Comassie blue staining, and Western blot analysis were carried out.
- For the *in vivo* studies, the human immortalized HEK293 cell line was used, and transfection with different DNA constructs was performed. Different stably silenced cell lines were generated.
- SDS-KCl precipitation assay was used to investigate the role of Spartan in the removal of DPCs
- BrdU comet assay modified with Proteinase K was carried out in order to investigate whether human Spartan has a role in DPC repair
- In order to investigate whether human Spartan facilitates immediate bypass of formaldehyde-induced DNA damage, DNA fiber assay was carried out

Results

Spartan possesses a DNA-dependent protease activity

Our *in vitro* experiments revealed that in the presence of DNA Spartan shows a DNA-dependent protease activity and can degrade itself similarly to other DNA-binding proteins such as Fan1, HLTF, and yRad5. Furthermore, we found that some proteins that have a high affinity to DNA such as RFC and RPA exhibit a concentration-dependent inhibitory effect on Spartan's DNA-dependent protease activity.

To check the DNA structural requirements for Spartan's protease activity, we tested various DNA structures for stimulation and found that several DNA structures could stimulate the activity at some level, but ΦX174 ssDNA stimulated the most, while double-stranded or nicked plasmid DNA stimulated quite weakly. In sum, our observations are consistent with a model of Spartan being targeted to exposed ssDNA, such as that found in case of fork stalling, where its protease activity can remove DNA-bound proteins.

Spartan is required for DPC removal

To reveal whether Spartan is involved in DPC removal *in vivo*, we monitored the DPC content of genomic DNA by separating the total genomic DNA into two fractions, a free-DNA- and a DPC-containing one, using a previously established SDS-KCl protein precipitation technique. We found that Spartan-silenced cells exhibited a somewhat higher amount of DPC even at the end of the 2 h formaldehyde treatment and retained higher amounts of DPC than the WT control cells during the 3 h and 6 h recovery time following formaldehyde treatment. From this experiment, we conclude that Spartan has a role in the removal of DPCs *in vivo*.

DNA-protein crosslink-specific Proteinase K-modified BrdU comet assay

The previously published BrdU comet assay (Single Cell Electrophoresis) protocol was modified in a way that the assay became highly specific for replication and DNA-protein crosslinks to reveal Spartan's role in the replication of DPC-containing DNA. Although formaldehyde exposure generates DNA-protein crosslinks, DNA-DNA crosslinks are also induced to a lesser extent. To distinguish between these two kinds of damage, Proteinase K treatment was introduced to eliminate the proteins crosslinked to DNA. Due to the removal of crosslinks, the free DNA can migrate in electrophoresis, resulting in a higher percentage of comet tail DNA. This modification enables the monitoring and measurement of DPCs at the single-cell level. Our result revealed that this method is reliable and highly DPC- and S-phase specific. Moreover, all formaldehyde concentrations used caused more prominent DPC accumulations in the Spartan-silenced HEK293 cell lines compared to control cells. From these results, we concluded that Spartan is required for the replication of DPC-containing DNA fragments; the lack of the protein leads to slower DPC removal; and Spartan's protease- or DNA-binding deficiency impairs highly the DPC-repair of replicating cells.

The PIP, UBZ, DNA-binding, and protease domains of Spartan contribute to the cells' resistance to DPCs

To investigate the role of the PIP, UBZ, SprT and DNA-binding domains of Spartan in DPC removal, BrdU comet assay, cell viability assay, and DNA fiber assay were carried out. The results of these assays showed that the DNA-binding domain- and protease domain-mediated activities of Spartan are significant determinants of the function Spartan has in the protection

against the genotoxic effects of formaldehyde-induced DPCs. However, the PIP and UBZ domains are required too.

Spartan facilitates immediate bypass of FA-induced DNA damage

One of the main hallmarks of replication stress is the abnormal slowing of replication fork movement, which can eventually lead to the stalling of the replication fork**.** Our next question was whether Spartan plays a role in the immediate bypass of DPCs, which can pose strong barriers to the movement of not only the replicative polymerases but the translesion synthesis polymerases as well. To monitor the speed of replication, we used the DNA fiber assay with which we can follow the elongation of the nucleotide analogue-labelled nascent DNA track at the single molecule level. To reveal whether Spartan can facilitate the bypass of formaldehyde-induced DNA damage, we compared Spartan-depleted and control cells and noticed that the lengths of the second tracks of Spartan-silenced cells are significantly shorter compared to those of control cells after formaldehyde treatment, reflecting a significantly stronger inhibitory effect of formaldehyde-induced damage on immediate bypass in the absence of Spartan.

Spartan and Rad18 act together in protecting the genome from DPCs

No particularly defined pathway has been assigned to DPC repair yet, and the discovery that the Spartan protease is required for the replication of DPC-containing DNA raised the question whether it constitutes an independent DPC repair pathway or acts together with other fork rescue pathways. To investigate whether Spartan acts together with Rad18 in DPC repair, we carried out cell viability, DNA fiber and BrdU comet assays. All three independent methods confirmed an epistatic relationship between SPARTAN and RAD18 in providing cellular resistance against DPC.

In summary, we showed that the purified human Spartan protein has a DNA-dependent protease activity degrading certain proteins bound to DNA. Our *in vivo* findings revealed that Spartan deficiency causes slower DPC removal and that Spartan's protease- or DNA-binding deficiency impairs highly the DPC repair of replicating cells as detected by a DPC-specific alkaline BrdU comet assay. Furthermore, we demonstrated, employing the DNA fiber method, that Spartan deficiency dramatically decreases the speed of replication forks under formaldehyde-induced replication stress, indicating a role for Spartan in the immediate replication of DPC-containing DNA.

We provided genetic evidence that human Spartan is required for facilitating DPC repair and replication of DPC-containing DNA and that it acts together with the RAD6-RAD18 DDT pathway.

Acknowledgment

I would like to thank my supervisor Dr. Lajos Haracska for the opportunity to conduct my experiments in his laboratory, for his support, and indispensable help.

I would like to thank Dr. Mónika Krisztina Mórocz and Róbert Tóth for their help in the experiments.

I am grateful to Dr. Tibor Pankotai for his thorough reading of my thesis in a short time.

I would like to thank Lajos Pintér, Dr. Péter Burkovics, Dr. Szilvia Juhász, Lili Hegedűs and Gabriella Tick for their help and support.

I am also thankful to Katalin Kovács for her technical help and to the Laboratory of Mutagenesis and Carcinogenesis Research and Delta Bio 2000 Ltd. for their help.

I am grateful to Dr. Miklós Erdélyi and the members of the Institute of Genetics of the Biological Research Centre for providing the background necessary for conducting my research.

I am grateful to Ágnes Tóth and Orsolya Frittmann for their help and support during hard times.

Last, but not least, I am thankful to my parents, husband, and friends for their support throughout my Ph.D. studies.

List of publications

MTMT identifier: 10055207
Total impact factor: 27,24

Publications supporting the dissertation:

Mónika Mórocz*****, **Eszter Zsigmond***, Róbert Tóth, Márton Zs Enyedi, Lajos Pintér, Lajos Haracska DNA-dependent protease activity of human Spartan facilitates replication of DNA–protein crosslink-containing DNA NAR. 2017 Apr 7; 45(6): 3172–3188. doi: 10.1093/nar/gkw1315, **IF: 11,14**
***** Shared first author

Caitlin A.L. Riddick, Peter D. Hunter, José Antonio Domínguez Gómez, Victor Martinez-Vicente, Mátyás Présing, Hajnalka Horváth, Attila W. Kovács, Lajos Vörös, **Eszter Zsigmond**, Andrew N. Tyler Optimal Cyanobacterial Pigment Retrieval from Ocean Colour Sensors in a Highly Turbid, Optically Complex Lake; REMOTE SENSING. 2019, 11(13), 1613; https://doi.org/10.3390/rs11131613, **IF:4,7**

Additional publications:

Sarolta Szentes, Nikolett Zsibrita, Mihály Koncz, **Eszter Zsigmond**, Pál Salamon, Zita Pletl and Antal Kiss I-Block: a simple *E. coli*-based assay for studying sequence-specific DNA binding of Proteins NUCLEIC ACIDS RESEARCH 2020 March 18; 48(5): doi: 10.1093/nar/gkaa014, **IF:11,4**

Conferences

Presentations

Zsigmond Eszter, Mórocz M, Toth R, Berczeli O, Dome L, Juhasz S, Haracska L: The role of human Spartan in the maintenance of genome integrity, Straub-napok, 2016 (Szeged, Hungary)

Zsigmond Eszter, Horvath H, Presing M: A Balaton és Kis-Balaton vízminőségének jellemzése a tápelemek és a fotoszintetikus pigmentek (a-klorofill és fikocianin) koncentrációja alapján, 13. Kolozsvári Biológus Napok, 2012 (Cluj-Napoca, Rumania)

Posters

Horvath H, Kovacs A, **Zsigmond E,** Voros L, Matyas K, Kobor I, Presing M: Estimation of cyanobacterial biomass based on the determination of their phycocyanin content, Interaction of Natureal and Social Processes in Shallow Lake Aleas International Conference, 2014 (Kaposvár, Hungary)

Horvath H, Kovacs AW, Voros L, **Zsigmond E**, Presing M: Extraction method of phycocyanin determination in filamentous cyanobacteria, Fresh Blood for Fresh Water Young Aquatic Science, 2013 (Lunz am See, Austria)
Horvath H, Kovacs AW, Matyas K, Sule G, Voros L, **Zsigmond E**, Presing M: Nitrogen fixation and phycocyanin content of phytoplankton in freshwaters, International Society of Limnology XXII. Congress, 2013 (Budapest, Hungary)