SUMMARY OF PH.D. THESIS

Molecular biological and genetic study of the sumoylation of *Drosophila melanogaster* p53

Pardi Norbert

Supervisor:

Dr. Boros Imre

Doctoral School of Biology

University of Szeged Faculty of Sciences and Informatics

Department of Biochemistry and Molecular Biology

Biological Research Center of the Hungarian Academy of Sciences

Department of Biochemistry

SZEGED
2011

Introduction

The p53 protein is one of the best-known tumor suppressors. Its mutations can be found in many different types of tumors (colon, lung, breast, oesophagus, liver, urinary bladder, ovaries, brain). Many times the main reason of tumor formation is not the presence of mutations in the p53 sequence, but the flaws in the p53-activating pathways. Activated p53 is able to inhibit the cell cycle, bring about apoptosis, and plays a vital role in the induction of cell differentiation. It is also well-known that wild type p53 inhibits angiogenesis in tumors by regulating genes involved in forming blood vessels. The cell cycle inhibiting function of p53 is achieved through its activity as a transcription factor and the protein is able to activate numerous apoptotic genes too.

Aims

The *Drosophila melanogaster* orthologue of human p53 was identified approximately at the same time by three American laboratories. Although the amino acid sequences of human p53 and Dmp53 are just partly identical they do bear numerous similar functions. This indicates that numerous p53-related biochemical pathways are conserved and it is sensible to study the functions of the protein in detail using the well-known model organism *Drosophila melanogaster*. Since p53 is a really important protein, all piece of information about it can be very interesting. Fly fruit - as an excellent multicellular model organism - offers a great possibility for the detailed biochemical and genetic observation of p53.

In order to clarify the role of p53 in flies I identified Dmp53-interacting partners by carrying out a yeast two-hybrid screen using a *Drosophila* cDNA library. Enzymes that play roles in the posttranslational modification called sumoylation were found to interact with Dmp53.

I wanted to identify which regions of Dmp53 interact with the DmUba2 (SUMO-activating enzyme), DmUbc9 (SUMO-conjugating enzyme) and DmPias (SUMO-ligase).

I investigated the genetic interactions between Dmp53 and the genes encoding the sumoylation enzymes.

Finally, my aim was to investigate the effects of the mutation altering the sumoylation site on the *in vivo* function of the protein.

Methods and Materials

In vitro recombinant DNS techniques
Nucleic acid preparation
Polymerase chain reaction, quantitative reverse transcription-coupled PCR
Northern analysis
Western analysis
Immunoprecipitation
Yeast two-hybrid experiments
GST-pulldown
Establishment of transgenic *Drosophila melanogaster* lines with P-element insertion and φC31 transgene integration system
In vivo functional studies with the UAS-GAL4 system
LOH assay
Staining of *Drosophila* larval tissues
Handling and transformation of *Drosophila* S2 cell culture
Measurement of luciferase activity

Results

1. The C-terminus of Dmp53 interacts with the sumoylation enzymes

By using yeast two-hybrid the C-terminus of Dmp53 was found to interact with the sumoylation enzymes. The C-terminus of the protein can be split functionally into two parts: oligomerization domain and basic domain. DmUba2 interacts with the basic domain of Dmp53, DmUbc9 interacts with both the oligomerization domain and basic domain. The full-length C-terminal fragment is required for the interaction with DmPias. The results of the yeast two-hybrid assays were verified by GST-pulldown. Only one site was found in the Dmp53 sequence that was fully identical to a consensus sumoylation site. The mutation of this site did not abolish the interactions between Dmp53 and the sumoylation enzymes.

2. Partial loss of sumoylating activity decreased the DNA repair activity of Dmp53

I tried to identify genetic interactions between Dmp53 and the sumoylation enzymes. In flies LOH assays are an established means of demonstrating the role played by Dmp53 in preserving genome stability. I carried out LOH assays based on detection of the recessive multiple wing hairs (Mwh) phenotype caused by the loss of the wild-type copy of *mwh*. To answer whether the decrease of the sumoylation activity has any effect on the mutator phenotype of *Dmp53* I used LOH assays. I generated flies simultaneously heterozygous for Dmp53, for the marker gene *mwh*, and for genes involved in sumoylation: *Su(var)2-10*, *lwr*, *smt3* or for both *lwr* and *smt3*. The homozygous *mwh* clones were counted on the wings of the adults. I found that partial loss of *Su(var)2-10*, *lwr* or *smt3* increased the number of wing clones. Thus, I concluded that partial loss of sumoylating activity enhanced the mutator phenotype of *Dmp53* suggesting that the decreased level of sumoylation may decrease the DNA repair activity of Dmp53.

3. Decreased sumoylation activity does not have any effects on the overexpression of Dmp53 in eyes

I examined the effect of decreased sumoylation activity on the overexpression of Dmp53 in eyes as well. I found that one copy of either *Su(var)2-10/ lwr/smt3/lwr-smt3* causes phenotypes in eyes similar to two copies.

4. Overexpression of Dmp53 in *Drosophila* causes severe phenotypes, and flies overexpressing the Dmp53^{K302R} protein die earlier than flies overexpressing Dmp53

For further study of Dmp53 I generated transgenic flies using P-element based techniques. I addressed the question whether overexpression of wild type or mutant Dmp53 causes any phenotypic differences. Using the UAS-GAL4 system I applied general and tissue-specific drivers to express the transgenes and analyzed the phenotypes. I used two strains overexpressing the Dmp53^{K302R} protein on the 2nd or the 3rd chromosome. Using either the 2nd or the 3rd chromosomal Dmp53-overexpressing strains I found that overproduction of the Dmp53^{K302R} protein causes more severe phenotypes than that of the wild type overexpressers. The phenotypes are stronger if general drivers are used. Interestingly, the phenotypes are stronger in males than in females. I generated Dmp53-overexpressing strains in which the transgenes are inserted into the same region of the 2nd or 3rd chromosome. In case the transgenes are inserted in this site-specific manner there is a smaller difference between the phenotypes caused by the overexpression of wild-type and mutant Dmp53 proteins.

We raised the question whether the stronger phenotypes are caused by the higher protein levels. In order to clarify this question I generated flies that carry two copies of the wild-type Dmp53-overexpressing transgene. With the overexpression of protein I found that more protein results in stronger phenotypes that are similar to that of the Dmp53^{K302R} overexpressers. In accord with this, the simultaneous overproduction of Dmp53^{K302R} and a dominant-negative form of Dmp53 (Dmp53^{H159N})

resulted in weaker phenotypes similarly to the wild-type Dmp53 overexpressers. These experiments show that the strength of the phenotypes are mainly determined by the Dmp53 levels.

5. K302R mutation stabilizes Dmp53

I used Western analysis to determine the amount of Dmp53 protein. I found that the protein is stabilized in the randomly inserted $Dmp53^{K302R}$-overexpressing flies and reach higher levels compared to the wild type Dmp53 overexpressers. I tried to detect SUMO-modified Dmp53 using the same samples but I was not able to detect higher migrating forms of the protein. In case of the overproduction of the mutant or wild type Dmp53 in the site-specific insertion containing strains I detected approximately similar levels of the proteins.

In the random insertion containing strains I determined the amount of gene products by Northern blot and Q-PCR. Similar levels of mRNA can be detected in the mutant and wild type Dmp53-overexpressing strains. On the basis of these experiments I concluded that Dmp53 protein is stabilized in $Dmp53^{K302R}$-overproducing flies and causes stronger phenotypes than that of wild type overexpressers.

6. SUMO modification of Dmp53 cannot be detected in protein samples either from flies or from S2 cells

A generally used method for the detection of sumoylation is using transfected cells. Wild type and mutant Dmp53 were transiently overexpressed in S2 cells and cell extracts prepared from these samples were analyzed by immunoblot. I was not able to detect any SUMO-modified form of Dmp53, however the overexpression of the proteins was successful. Immunoprecipitations were also carried out by using higher amount of S2 cells, but no sumoylated Dmp53 was detected, although these experiments were repeated several times. Cotransfection experiments were also performed in which Dmp53 and SUMO were overproduced simultaneously. No

modified Dmp53 forms were detected, although these methods are wide-spread for investigating posttranslational modifications of proteins.

7. Dmp53 is located in the nucleus and the K302R mutation does not alter its cellular localization

Sumoylation has numerous well-known biological functions. Many times the unmodified and sumoylated forms of proteins are localized in different compartments of the cells so the processes of sumoylation/desumoylation are essential for the transport of the given protein between the cytoplasm and the nucleus. Immunostained *Drosophila* tissues revealed that both the wild type and the K302R mutant form of the protein are localized in the nucleus. This suggests that the transport of Dmp53 is not or not exclusively influenced by the SUMO-modification.

8. The K302R mutation does not influence the transcription activation ability of Dmp53

Another important role of sumoylation is the modification of transcriptional activity of the target protein. I examined the transcriptional activating capacity of the wild type and K302R mutant Dmp53 by performing luciferase assays using S2 cells. No obvious difference was found in the transactivation ability of Dmp53 and $Dmp53^{K302R}$.

List of publications

Daxx-like protein of Drosophila interacts with Dmp53 and affects longevity and Ark mRNA level.
Bodai L, Pardi N, Ujfaludi Z, Bereczki O, Komonyi O, Balint E, Boros IM
J Biol Chem. 2007 Dec 14;282(50):36386-93.
IF: 5,854

TATA binding protein associated factor 3 (TAF3) interacts with p53 and inhibits its function.
Bereczki O, Ujfaludi Z, Pardi N, Nagy Z, Tora L, Boros IM, Balint E
BMC Mol Biol. 2008 Jun 12;9:57.
IF: 4,485

In vivo effects of abolishing the single canonical sumoylation site in the C-terminal region of Drosophila p53
Pardi N, Vamos E, Ujfaludi Z, Komonyi O, Bodai L, Boros IM
Acta Biol. Hung. 62 (4), 2011
IF: 0,636

Conference presentations and posters

30th FEBS Congress- 9th IUBMB Conference
N. Pardi, Z. Újfaludi, L. Bodai, O. Bereczki, É. Bálint and I. M. Boros
Identification and characterization of p53-interacting proteins and target genes in *Drosophila melanogaster* (poster)

20th International Congress of Biochemistry and Molecular Biology
N. Pardi, L. Bodai, É. Bálint and I. M. Boros
The role of sumoylation in *Drosophila melanogaster p53* function (poster)

8th International Conference on Drosophila Heterochromatin
N. Pardi, L. Bodai, O. Komonyi, Z. Újfaludi, O. Bereczki, É. Bálint, and I. M. Boros
Daxx-like protein of *Drosophila* interacts with Dmp53 and effects longevity and Ark mRNA level (poster)

32nd FEBS Congress, Molecular Machines
O. Bereczki, Z. Ujfaludi, **N. Pardi**, L. Tora, I. M. Boros, E. Balint
Evolutionarily conserved interaction of P53 with Drosophila melanogaster Bip2/mammalian TAF3 (poster)

12th Symposium of the Hungarian Biochemical Society
Bereczki O., Újfaludi Z., **Pardi N.**, Boros I. M., Bálint É.
Analysis of the TAF3-p53 interaction (poster)

11th Symposium of the Hungarian Biochemical Society
Bereczki O., **Pardi N.**, Boros I. M., Bálint É.
Functional analysis of the p53-TAF3 and p53-Bip2 interactions (poster)

9th *Symposium of the Hungarian Biochemical Society*
Újfaludi Z., Bereczki O., Cziglédszky A., **Pardi N.**, Bálint É., Boros I. M.
Identification of regulating proteins for Dmp53 (=*Drosophila* p53) and by Dmp53 regulated genes (poster)

8th Symposium of the Hungarian Biochemical Society
A. Cziglédszky, **N. Pardi**, É. Balint, I. M. Boros
Identification of *Drosophila* p53 interacting proteins using yeast two-hybrid screen (poster)

Straub days, 2007, Szeged
Norbert Pardi, Orbán Komonyi, László Bodai, Újfaludi Zsuzsanna, Éva Bálint, Orsolya Bereczki, Imre M. Boros
***Drosophila p53*- a model for the better understanding of human p53 function (presentation)**

Acknowledgement

I would like to thank my supervisor, Dr. Boros Imre for his professional guidance, valuable advices, and for reading and commenting on my thesis. The technical help and practical advices of Ökrösné Katalin were great contributions to my research, for which I am very grateful. I would also like to express my gratitude to the members of our group, who provided a nice atmosphere during my work. Last, but not least I would like to thank the support of my family that enabled me to concentrate on my work.