THESIS BOOK

The functional investigation of the interaction between TATA-associated factor 3 (TAF3) and p53 protein

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Introduction

The p53 tumour suppressor gene and its protein product have became the objects of intense study since their discovery in 1979, because in most human tumours confirmed that p53 is mutated or the p53 pathway is deficient. In Li-Fraumeni syndrome, mutations of p53 occurring in the germ line lead to tumor formation in young ages with high familiar frequency.

The p53 is a transcription factor, which has versatile roles in genome protection after DNA damage. Mutations of p53 result in the accumulation of DNA damages and unlimited proliferation of the injured cells.

In unstressed cells the level of p53 is very low by a proteasome-mediated degradation. In this progress the MDM2 protein plays a key role, which binds to the N-terminal region of p53 protein and by its ubiquitin ligase activity to degrade the p53. The p53 mediates the expression of own negative regulator, MDM2 protein in a autoregulatory feedback loop, because the expression of MDM2 activated transcriptionally by p53.

Upon DNA damage, p53 is phosphorylated by Chk1 (checkpoint kinase 1) and Chk2 (checkpoint kinase 2) kinases leading to the rapid elevation of p53 level and its activation as transcription regulator. Activated p53 is transported to the nucleus where it functions as a transcription factor inducing or repressing its target genes, activates cell cycle block and accompanying DNA repair or apoptosis and inhibiting the angiogenesis.

In vertebrates, the p53 gene has two other homologs, p63 and p73. Both of them express several isoforms through alternative promoter usage and alternative splicing (mRNA editing). The high level of sequence similarity between p63, p73 and p53 proteins, allows p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis. The p53 protein execites its functions in these processes in interaction with numerous other proteins and the exploration of these interactions is an important task, on which members of our group began to work several years ago.

Previously our research group identified in yeast two hybrid experiments (Y2H) several interacting partners of *Drosophila melanogaster* p53 (Dmp53).

One of our identified partners, the TATA box-binding protein-associated factor, TAF3, which interaction is evolutionarily conserved.

The mouse TAF3 (mTAF3) decreased specifically and dramatically the transcriptional activity of p53 and reduced in a lesser extent the p53 protein level, without any change in p53 mRNA level. The mTAF3 did not influence the transcriptional activity of p53 related proteins but in the presence of endogen p53 it affected their protein level.

The investigation of functional consequence of interactions contribute to detailed analysis of the functions of p53 and identification of its target genes may lead to a better understanding of tumor formation and development of cancer therapies.

Aims

1. To characterize the interaction between Dmp53 and DmTAF3. To analyse interaction between human p53 family members (p53, p73α and p73β) and the mouse homologue of TAF3 both the *in vivo* and *in vitro*.

2. To examine the functional significance of TAF3 interaction with p53 and p53 related genes (p73α and p73β).

3. To analyse the function of the PHD domain, located on the C-terminal region of TAF3.

Methods

- Detection of protein-protein interactions by yeast two hybrid system
- Proteins expression and purification in bacteria in GST fusions
- GST pull down assay
- *In vitro* translation
- Recombinant DNA techniques: polymerase chain reaction (PCR), restriction digestion, ligation, DNA purification
- Maintenance of mammalian cell lines, transient transfections
- Determination of transcriptional activity by reporter gene analysis luciferase activity determination
- Co-immunoprecipitation
- Western blotting
- Quantitative real time PCR
- *In vitro* ubiquitination assay
- *In vivo* ubiquitination assay

Results

The TAF3 protein is a new interacting partner of p53 protein

To identify the interacting partners of *Drosophila* p53 (Dmp53), we screened *Drosophila* embryonic cDNA library by yeast two hybrid (Y2H) assay. Among several identified cDNA clones one (5# clone) contained the coding region for the 514-924 amino acids of DmTAF3. Another clone cDNA (11# clone) encoded amino acids 738-1061 of DmTAF3. By comparing of two clones we concluded that the region which is responsible for Dmp53 interaction must lie between amino acids 738-924 of DmTAF3. To identify the regions of Dmp53 responsible for the interaction we tested the binding of different parts of p53 to the DmTAF3 region encoded by clone 5# in Y2H experiment. We found that the DNA-binding domain of Dmp53 showed only a weak, but the C-terminal part showed a very strong interaction with DmTAF3.

Further analysis of the interaction of DmTAF3 and Dmp53 C-terminal region showed that the oligomerization domain alone was able to interact strongly with DmTAF3. However, the regulatory domain alone showed only a weak interaction with DmTAF3. These results indicate that DmTAF3 is able to interact with Dmp53 through multiple binding sites. We further confirmed the interaction between DmTAF3 and Dmp53 by using *in vitro* system (pull down assay) as well.

The interaction between TAF3 and p53 family members is evolutionarily conserved

Next we examined, if the DmTAF3 interacts with human p53 as well. Our experiments showed a very strong interaction between DmTAF3 and p53 proteins. So the regions responsible for interaction with DmTAF3 are conserved and present in both human and Dmp53. Furthermore, we detected and verified the interaction between mouse TAF3 (mTAF3) and p53 by *in vitro* assays and *in vivo* in human cells as well. Similarly, we could confirm interaction between p73α and p73β and mTAF3 both by Y2H and *in vitro* binding assay. Based on these data we concluded that the interaction between TAF3 and p53 is evolutionarily conserved.

Next, we asked that, what is the functional consequence of these interactions. In order to answer this question we studied the effect of mTAF3 on the transcriptional activity of p53, p73α and p73β proteins.

mTAF3 decreases the transcriptional activity and protein level of p53

Using transient reporter gene assay we found that mTAF3 decreased dramatically the transcriptional activity of endogen p53 in U2OS cells. The activity of an exogen p53, synthetised from a transfected template was affected by TAF3 to a much lesser extent.

Overexpression of mTAF3 resulted in a small decrease in the p53 protein level. The results suggest that the decrease of protein level cannot be responsible for the observed reduction of p53 transcriptional activity.

Further we could not find any change in the p53 mRNA level after mTAF3 overexpression. Therefore we conclude that mTAF3 does not to influence the synthesis or the stability of p53 mRNA. In HeLa cells the degree of decrease in exogen p53 protein level was comparable to the inhibition of p53 transcriptional activity.

In summary, based on these results we concluded that the mTAF3 is able to inhibit the transcriptional activity of p53, and, in a lesser extent is also reduces the p53 protein level, without any change in p53 mRNA level.

mTAF3 does not influence the transcriptional activity of p53 related proteins but alters their protein level

The mTAF3 could not influence the transcriptional activity of p53 related proteins either in HeLa cells which contain only a low level of endogenous p53 or in Saos2 cells which do not contain any endogenous p53. But in the presence of endogenous p53 in U2OS cells we found that the protein level of p73α decreased and that of p73β increased owing to mouse TAF3. Consequently the transcriptional activity and protein level of p53 related proteins changes differently in the presence or absence of p53.

The role of mTAF3 PHD domain

Could the mTAF3 function as an E3 ubiquitin ligase?

Both the *Drosophila* and mouse TAF3 proteins contain a HFD domain in the N-terminal and a PHD domain in the C-terminal region. Interestingly, these domains are not conserved in different TAF3 homologues, since in contrast of the mouse and *Drosophila* proteins, in the human homologue no PHD domain has been found experimentally in HeLa cells. Because of the C-terminal region of p53 protein is responsible for the interaction between p53 and TAF3, therefore we investigated if the inhibitory effect is explained with the C-terminal modification of p53 by TAF3. We examined the ubiquitination from between possible modifications of p53, because of domain structure of mTAF3 protein and several papers reported on the E3 ubiquitin ligase activity of PHD domain containing proteins.

Therefore we wanted to know, if the PHD domain of mTAF3 could play a role in the degradation of p53 protein. We examined this question by *in vitro* and *in vivo* ubiquitination assays. These experiments, however could not verify unambigously the E3 ubiquitin ligase activity of TAF3.

The PHD domain-mutant mTAF3 does not inhibit the transcriptional activity of p53

To examine further the possible role of PHD domain, we investigated the effect of PHD domain-pointmutant (H893A) and PHD-deletion-mutants (K620Stop, V849Stop) mTAF3 on the transcriptional activity of p53. In the case of H893A pointmutant, where the mutation is in the central histidin of the Cys4-His-Cys3 Zn^{2+}-finger type PHD domain, the effect is the same than in the case of V849Stop deletion-mutant, which lacks the full PHD domain. We found that in contrast to the wild type mTAF3, which dramatically decreased the transcriptional activity of p53, the mutants were not able to induce the inhibitory effect. This loss of the inhibitory effect correlated with the size of the missing sequence from the mTAF3 protein and the PHD domain.

So it is conceivable, that not the presence of the PHD domain, rather the loss of the p53 interacting region of TAF3 cause the disappearance of the inhibitory effect on transcriptional activity.

The PHD domain-mutant mTAF3 does not affect the protein level of p53

We examined the effect of the PHD domain-mutant mTAF3 on the protein level of p53. As expected, we found that the wild type mTAF3 decreased slightly the p53 protein level, but the PHD domain-mutant mTAF3 had no effect on the p53 content.

The hTAF3 which does not contain PHD domain inhibits the transcriptional activity of p53 and of p53 related proteins

The presence of the PHD domain in the C-terminal part of the human TAF3 protein was not confirmed so far in HeLa cells.

This offered the possibility to test whether in the absence of PHD domain TAF3 is able to cause any change on the transcriptional activity of p53 and of p53 related proteins.

We found that the p73α was affected by hTAF3 similarly as with mTAF3, such as no effect on the transcriptional activity was detected. However, p73β was decreased only by the human homologue but not by mouse TAF3. In the case of p53, the decreased transcriptional activity was observed independently of the PHD domain. It is therefore we can conclude that the presence or absence of the TAF3-p53 interaction region will determine whether the inhibitory effect of TAF3 on p53 transcriptional activity is manifested or not.

Summary

My results and my conclusions are:

1. The identification of DmTAF3, as a new interacting partner of Dmp53 protein.
2. Detection and demonstration of the interaction of DmTAF3 with human p53 and p53 family members, p73α and p73β.
3. Demonstration of the evolutionarily conserved interaction between the TAF3 and p53 proteins both *in vitro* and *in vivo*.
4. The mouse TAF3 inhibits the transcriptional activity of p53, and, in a lesser extent reduces the p53 protein level.
5. The mTAF3 can not influence the transcriptional activity of p53 related proteins, but depending on the presence of endogen p53 it alters the protein level of p73α and p73β.
6. The effect of mTAF3 on p53 transcription is related to the presence of the PHD domain.
7. In contrast to the wild type mTAF3, in the PHD domain-point-mutant and deletion-mutants mTAF3 has no effect on the p53 protein level.
8. The human TAF3, which does not contain the C-terminus PHD finger domain, inhibits the transcriptional activity of p53 and p73β as well.

Publications included in the thesis

1. **Bereczki O**, Ujfaludi Z, Pardi N, Nagy Z, Tora L, Boros IM, Balint E. *TATA binding protein associated factor 3 (TAF3) interacts with p53 and inhibits its function*. BMC Mol Biol. 2008, 9:57. IF: 3,5.

2. Bodai L, Pardi N, **Ujfaludi Z**, Bereczki O, Komonyi O, Balint E, Boros IM. *Daxx-like protein of Drosophila interacts with Dmp53 and affects longevity and Ark mRNA level.* J Biol Chem. 2007; 282(50): 36386-93. IF: 5,808