Summary of the Ph.D dissertation

Analysis of the mechanism by which dADA2 factors contribute to the specificity of dSAGA and dATAC histone acetyltransferase complexes

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INTRODUCTION

The DNA sequence is packed by histone and non-histone proteins into chromatin. Histones are small basic proteins consisting of a globular domain and a flexible, charged amino- and carboxy-terminus (histone "tail"). The histone tails are highly conserved across various species and are subjected to extensive post-translational modifications (PTMs) (1). The histone protein acetylation represents one of the most studied PTMs, a highly dynamic process regulated by opposing action of histone acetyltransferases (HAT) and histone deacetylases (HDAC) (2-4).

The GCN5 (general control nonderepressed 5) protein is the catalytic component of several HATs (5) that also share ADA2 (alteration/deficiency in activation 2) adaptor proteins. The ADA2 proteins have been reported to play an important role in transcriptional initiation site selection, interact with basal transcription factors and potentiate the HAT activity of GCN5 (6, 7).

In *Drosophila melanogaster* there are two *Ada2* genes referred to as *dAda2a* and *dAda2b*. The two dADA2 proteins are similar in containing in their N terminal region a ZZ domain followed by a SANT domain and three less conserved regions designated as ADA boxes with chromatin binding functions as was recently identified (8-10). dADA2 proteins participate strictly in distinct complexes and are not able to functionally substitute each other (11). dADA2a is present in a smaller complex (dATAC) responsible for acetylation of histone H4 at lysine K5 and K12, while dADA2b is present in a larger (dSAGA) complex which targets H3 K9 and K14 for acetylation (9, 11, 12). Therefore, the ADA2 coactivator proteins together with different components of histone acetyltransferase complexes are major determinants in specifying the substrate preference and also gene-specific targeting.

Past decade of extensive studies on histone modifications have demonstrated their important role in regulating different physiological processes. Inappropriate or altered gene expression patterns due to abnormal PTMs can lead to many human diseases, including cancer and neurodegenerative disorders (13). Moreover, histone covalent modifications have also been correlated with the control of learning and memory functions (14). Thus elucidating the role of HATs and HDACs in orchestrating proper cellular development and differentiation will enable the therapeutic usage of modulators aimed at preserving the physiological equilibrium of acetylation (15).

RESEARCH OBJECTIVES

In *Drosophila melanogaster* dADA2a and dADA2b proteins are subunits of dATAC and dSAGA HAT complexes, respectively. Despite the fact that the two dADA2 proteins contain similar conserved domains and interact with similar partners they function in distinct HAT complexes. These observations made us interested in determining what particular domain within dADA2 proteins is responsible for their interaction with different transcriptional coactivators and the dGCN5 acetyltransferase. The regions that specify the association of dADA2a and dADA2b proteins to one or the other HAT complexes were investigated by:

- Constructing transgenes that direct the expression of chimeric dADA2a/2b or dADA2b/2a proteins;
- Assessing their *in vivo* function by expressing the chimeric proteins in *D. melanogaster ada2a*^{d189} or *ada2b*^{d842} mutants;

- ❖ Determining the histone acetylation pattern on polytene chromosomes of both of the *ada2* null animals expressing the chimeric transgenes;
- ❖ Comparing the expression patterns of selected genes from *ada2a*^{d189} or *ada2b*^{d842} null mutants to those expressing the chimeric proteins.

Furthermore, two dADA2b protein isoforms have been reported in *Drosophila melanogaster* which might extend the functional complexity of dSAGA. The two dADA2b isoforms are identical at the N-terminal region but differ in their C-terminus. Both isoforms contain a Zinc finger and a SANT domain in their N-terminal region and two ADA boxes. Only the long dADA2b isoform contains a third ADA box in its C-terminal part. These observations made us interested to investigate whether the two isoforms are subunits of the same HAT complex or there are distinct HAT complexes with different dADA2b isoforms.

MATERIAL AND METHODS

- ❖ Molecular cloning methods, Gateway technology
- ❖ Cloning of chimeric DNA sequences
- ❖ Constructs preparation for co-immunoprecipitation
- ❖ *Drosophila* S2 cell transient transfection, protein expression and purification
- ❖ Co-immunoprecipitation
- ❖ Western analysis
- ❖ *Drosophila* genetic analysis
- ❖ Generation of transgenic *Drosophila* lines
- ❖ *In vivo* functional studies with UAS-Gal4 system

- ❖ Immunofluorescence of polytene chromosomes
- ❖ RNA isolation, cDNA synthesis
- ❖ Quantitative Reverse-Transcriptase PCR

RESULTS

1. Engineering the chimeric transgenes

In *D. melanogaster* GCN5 represents the catalytic subunit of dSAGA and dATAC HAT complexes and together with dADA2a and dADA2b type adaptor proteins modulate the HAT activity and specificity of the two HAT complexes. Functional studies of the two dADA2 proteins have identified that, while loss of dADA2a function affects the acetylation of histone H4 at lysines K5 and K12 (12), the *ada2b*^{d842} null mutation abolishes the H3K9 and K14 acetylation (11). Subsequently, detailed analysis of the dATAC complex revealed, in addition to dGCN5, the presence of another HAT catalytic subunit, dATAC2 with a pivotal role in H4K16 acetylation in embryos. Thus we questioned whether the histone H4 acetyltransferase activity of dATAC can be attributed to dGCN5 or to another HAT of the complex. Immunoblots of total protein extracts of wild-type and *gcn5*^{E333st} late third-instar larvae revealed a significantly reduced level of histone H3 and H4 acetylation at K12 and K14 when the function of the dGCN5 catalytic subunit is altered.

In light of the above and earlier data we wondered on the possible roles of dADA2 factors in dATAC and dSAGA complexes. In order to gain more information whether the two dADA2 factors might modulate the GCN5 specificity within dATAC or dSAGA complexes, recombinant chimeric dADA2's

were constructed by joining PCR-generated DNA fragments corresponding to functional domains of one and the other dADA2 proteins. We transiently transfected the plasmids expressing the chimeric constructs into *Drosophila* S2 cells and verified by western blot that each plasmid directed the synthesis of a chimeric protein with the expected size.

The *in vivo* characterization of the chimeras was carry out by generating transgenic lines where coding region of chimeric proteins were inserted into a P-element containing vector allowing site specific insertion into *Drosophila* genome according to *ϕC31* integrase system. The chimeric transgenes were then tested for their ability to rescue $ada2a^{d189}$ or $ada2b^{d842}$ mutation.

2. *Expression of dADA2 chimeric proteins partially rescues $ada2a^{d189}$ or $ada2b^{d842}$ mutation*

The animals carrying $ada2b^{d842}$ mutation were previously characterized as P5 pupa stage lethal (11). Out of the four dADA2 chimeric transgenes tested in $ada2b^{d842}$ mutant background, only the animals expressing pUAS-*dAda2a2b* transgenes showed partial but relevant rescue. Under the control of Actin-GAL4 driver more than 50% of the pUAS-*dAda2a2b* transgene-carrier animals could complete the P5 stage and develop as far as P14 stage. However, we could observe a two day delay in growth compared to the heterozygous siblings. Moreover, a small percentage of the animals could complete the developmental stage and emerged as adults, although died within 12 hours with the wings still folded. The expression of pUAS-*dAda2b2a* chimeric transgenes in $ada2b^{d842}$ mutant background failed to show any rescue of the phenotypes in contrast to the pUAS-*dAda2a2b* transgene. As control the expression of pUAS-*dAda2b* transgene partially rescues $ada2b^{d842}$ P5 lethality

and supports the development to P14 stage for 80% of the animals. A full rescue with this transgene is not expected since *dAda2b* cDNA used to generate pUAS-*dAda2b* transgenic lines encoded the short isoform of dADA2b protein. On the contrary, the expression of pUAS-*dAda2a* transgene in *ada2b*^{d842} mutant background resulted in 50% of the animals reaching P12 stage with phenotype characteristics similar to those of escaper *ada2b*^{d842} mutants.

The chimeric transgenes were next investigated for their potential to rescue the characteristic features of *ada2a*^{d189} null mutation. The *ada2a*^{d189} is a null allele mutation and homozygotes can survive for up to 2 weeks in L3 larval stage but fail to undergo prepupal contraction or form malformed brownish pupae with incompletely everted anterior spiracles (12). Expression of pUAS-*dAda2a* transgene resulted in complete rescue, while pUAS-*dAda2b* transgene had no effect on the phenotype. When we tested the rescue ability of pUAS-*dAda2b*^{S}*2a* and pUAS-*dAda2b*^{M}*2a* chimeric transgenes we could observe rescue of L3 lethality in more than 20% of the animals. In contrast to the *ada2a*^{d189} mutants that showed arrested development and persisted in the L3 larval stage for up to 2 weeks, the expression of *dAda2b*^{S}*2a* and *dAda2b*^{M}*2a* transgenes helped to rescue this developmental defect and the animals reached the P5 stage in 4 days. However, a two days delay in their development was recorded compared to that of wild type. Either the pUAS-*dAda2b*^{L}*2a* or pUAS-*dAda2a2b* transgene expressions in *ada2a*^{d189} mutant background presented no significant rescue of the phenotype relative to that of pUAS-*dAda2a* transgene.

Taken together the rescue experiments suggest that the C-terminal region of dADA2a and dADA2b proteins has an important role in determining the

rescue ability of the chimeric transgenes in *ada2a*^{d189} and *ada2b*^{d842} mutant animals, respectively. The high homology between the two proteins helped in preserving a proper folding of the chimeric proteins similar to the wild type proteins and partially restoring one or the other dADA2 functions.

3. *The acetylation level of histones H3 or H4 at specific lysine residues is restored by expression of dADA2 chimeric proteins*

To emphasize the outcome of the rescue experiments, polytene chromosome stainings were performed to determine whether the expression of the pUAS-*dAda2b*^{S}*2a*, pUAS-*dAda2b*^{M}*2a* and pUAS-*dAda2a2b* transgenes are able to restore the loss of acetylation as a result of *ada2a*^{d189} or *ada2b*^{d842} mutation.

In *ada2b*^{d842} mutant animals the H3K9 and K14 acetylation was restored by the expression of pUAS-*dAda2a2b* transgene, while the pUAS-*dAda2b*^{S}*2a*, pUAS-*dAda2b*^{M}*2a* or pUAS-*dAda2b*^{L}*2a* chimeric transgenes had no detectable effect on either H4K9 or K14 acetylation. Immunoblots developed with the same antibodies corroborated the results of the polytene chromosome stainings.

Expression of pUAS-*dAda2b*^{S}*2a* or pUAS-*dAda2b*^{M}*2a* transgenes in *ada2a*^{d189} mutants recovered the intensity of banding pattern for H4K5 and K12 acetylation. Moreover, an improvement of the polytene chromosomes structure was observed for *ada2a*^{d189} null mutants expressing the chimeric transgenes. The immunoblotting of total protein extract from *ada2a*^{d189} mutants containing the dADA2b^{S}2a or dADA2b^{M}2a chimeric proteins with antibodies specific for H4K5 and K12 acetylation corroborated the results of the polytene chromosome stainings.

Thus, the *in vivo* changes at the acetylation levels observed for histone H3 and H4 lysines suggest that the chimeric proteins can partially substitute the function of one or the other dADA2 protein within the HAT complexes.

4. *Chimeric dADA2 proteins did not improve expression of selected genes affected by ada2a^{d189} or ada2b^{d842} mutation*

Recently it has been reported that the expression of a small set of genes are influenced by *ada2b^{d842}* mutation whereas a considerable number of genes are affected by the loss of function of dATAC-specific subunit (16, 17). Based on the results of the rescue experiments and the polytene chromosomes immunostainings we can hypothesize that the expression of the chimeric transgenes in *ada2* mutants might determine gene expression changes as well. To study the molecular consequences of the chimeric transgene expression we compared the mRNA levels of selected genes from wild type and chimeric transgene expressers by Quantitative Real-Time PCR. Among the genes affected by *ada2b^{d842}* mutation, we selected to analyse those which were found to be either down-regulated like *Sugarbabe* (Sug) and *Cap'n'collar* (Cnc), or up-regulated like *Frost* (Fst) and *Hus 1-like* (Hus-1) (17). From *ada2a^{d189}* dependent genes we chose few belonging to the group of *Halloween* genes involved in the ecdysone biosynthesis pathway: *Phantom* (Phm), *Spookier* (Spok) and *Shadow* (Sad) which were found to be downregulated in *ada2a^{d189}* mutants (16). Surprisingly, by comparing the mRNA levels of *ada2* mutants and chimeric transgene-carrier *ada2* mutant animals we did not detect significant changes in mRNA level corresponding to either of the above mentioned genes. The failure to detect any change in mRNA level of the

selected genes in either one or the other *ada2* mutants expressing chimeric transgenes might be the result of the partial rescue.

5. In vivo interaction between dADA2b isoforms, dADA3 and dp53

The *dAda2b* gene in *D. melanogaster* gives rise to two protein isoforms (dADA2bS and dADA2bL), which might extend the functional complexity of the dSAGA. The two isoforms are identical in their N-terminal 330aa region that harbours the ZZ zinc finger and SANT domains, characteristic for all known ADA2 proteins, and two so-called ADA boxes (8). The presence of two dADA2b isoforms and their co-fraction with subunits of dSAGA HAT complex might suggest that the short and the long dADA2b isoforms are subunits of either the same or similar multiprotein complexes.

Experiments were performed to analyse whether the dADA2 isoforms can interact with each other and whether they interact with dADA3, another component of dADA2b-containing HAT complexes. Previous studies have demonstrated that mutations of the *dAda2b* gene affected dp53 functions (11) and a physical interaction between dADA2b and dp53 was also shown (9). Hence, we were interested to learn if dADA2b isoforms displayed differences in interaction with the tumour suppressor dp53.

To detect protein-protein interactions N-terminal FLAG or HA epitope tagged dADA2b isoforms were transiently expressed in *Drosophila* S2 cell line. Co-immunoprecipitation of dADA2bS with dADA2bL and vice versa indicated *in vivo* physical interaction between the two isoforms. Furthermore, both dADA2b isoforms co-immunoprecipitated with dADA3 and both, dADA2bS and dADA2bL, co-immunoprecipitated with dp53. Taken together, the tested protein-protein interactions did not reveal different abilities between the two

dADA2b isoforms to participate in interactions, but demonstrated the possibility of dimer formation between dADA2bS and dADA2bL proteins. Moreover, by interacting with dADA3, subunit of dSAGA complex, we can hypothesize that both dADA2b isoforms are components of the same or similar HAT complexes. A possible role of the two isoforms in different dp53-mediated pathways can also be considered since both dADA2b isoforms interacted with the tumour suppressor dp53.

SUMMARY

In this dissertation several lines of evidence are provided for the role of the C-terminal domain of *Drosophila* dADA2 adaptor proteins in regulating the specificity of dSAGA or dATAC complexes *in vivo*. Using a series of domain swap chimeras, in which different fragments of dADA2b and dADA2a were mutually exchanged, we examined the importance of particular regions for the specificity of the HAT complexes. We tested the *in vivo* functions of the chimeric dADA2 proteins by determining the phenotypical rescue and histone modifying abilities of dADA2a/dADA2b chimeras in $ada2a^{d189}$ and $ada2b^{d842}$ null mutants. We found that Actin-Gal4 driven expression of the chimeric dADA2 proteins had a partial phenotypical rescue effect for one or the other *ada2* mutants. Immunostaining of *Drosophila* polytene chromosomes and western blot analysis revealed a significant restoration of the lost acetylation on H4K5 and K12 or H3K9 and K14 in *ada2* mutants carrying chimeric transgenes. These findings promote the hypothesis that the C-terminal domain of the dADA2 proteins plays an important role regarding the particular incorporation of dADA2 proteins into dSAGA or dATAC type complexes. Furthermore, the ability to use dADA2 chimeric proteins to rescue and

examine developmental processes provides a strategy for associating specific structural domain of dADA2 proteins with functional specificity.

Biochemical and genetic evidences revealed the existence of two isoforms of dADA2b protein expressed in different quantities during fly development (Pankotai et al., 2013b). The experimental data presented in this thesis establish that both dADA2b proteins interact with each other and co-immunoprecipitated from S2 cells with dADA3, as well as with the tumour suppressor dp53. These findings show that both dADA2b proteins are functional and support the possibility that either both dADA2bL and dADA2bS can share the same HAT complex or they are subunits of different SAGA complexes. In either way their interaction with dp53 might suggest a role in the apoptotic pathway.

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STATEMENT

As the corresponding author of the paper by Pankotai et al. entitled „*Functional characterization and gene expression profiling of Drosophila melanogaster short dADA2b isoform-containing dSAGA complexes*" Genomics 2013, 14:44, hereby I testify that the data presented in that paper on the interactions of dADA2bS, dADA2bL, dADA3 and dp53 proteins resulted from experiments performed by Ecaterina Edith Vamoş and those data neither have been nor will be used in any other dissertation than that of her.

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LIST OF PUBLICATIONS

Publications related to the thesis

- **Vamos E. E.**, Boros I. M. - *The C-terminal domains of ADA2 proteins determine selective incorporation into GCN5-containing complexes that target histone H3 or H4 for acetylation.*
FEBS Letters 2012 September 21; 586 (19):3279-86.
IF: 3.538

- Pankotai T., Zsindely N., **Vamos E. E**., Komonyi O., Bodai L., Boros I. M. - *Functional characterization and gene expression profiling of Drosophila melanogaster short dADA2b isoform-containing dSAGA complexes.*
BMC Genomics 2013 January 22; 14 (1):44.
IF: 4.07

Other publications

- Pankotai T., Ujfaludi Z., **Vamos E. E.**, Suri K., Boros I. M. - *The dissociable RPB4 subunit of RNA Pol II has vital functions in Drosophila.*
Mol. Genet. Genomics 2010 January 283 (1):89-97.

IF: 2.635

- Pardi N., **Vamos E. E**., Ujfaludi Z., Komonyi O., Bodai L., Boros I. M. - *In vivo effects of abolishing the single canonical sumoylation site in the C-terminal region of Drosophila p53.*
Acta Biol. Hung. 2011 December 62(4):397-412.

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