SUMMARY OF THE PH.D. THESIS

DEVELOPMENTAL-STAGE-SPECIFIC REGULATION OF THE POLYUBIQUITIN RECEPTORS IN *DROSOPHILA MELANOGASTER*

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INTRODUCTION

In the maintenance of cellular homeostasis the ubiquitin-proteasome system (UPS) has pivotal role. This system is responsible for the rapid and selective degradation of short-lived proteins that have mostly regulatory role in the cell. In addition, UPS is involved in the quality control of the cellular proteins by degrading the irreversible denatured proteins. The first step of this degradation system is catalyzed by the ubiquitin ligase enzyme cascade, which recognizes the proteins intended for intracellular degradation and marks them by covalently attaching a polyubiquitin chain to a lysine residue of the substrate protein. This degradation signal is recognized by polyubiquitin receptors, which bind the polyubiquitylated proteins and target them for degradation to the 26S proteasome. The 26S proteasome, a large multiprotein complex is built up from two subcomplexes: the central catalytic core is responsible for the proteolytic degradation, while the 19S regulatory complexes recognize the polyubiquitin degradation signal, specifically bind these substrate proteins, deubiquitylate and unfold the substrate proteins and feed them into the central catalytic particle.

One of the most controversial fields of proteasome research is the way in which polyubiquitin receptors fulfill their job. Although a large amount of information was collected by molecular and genetic studies in the yeast *Saccharomyces cerevisiae*, the mechanism how these receptors recognize their specific substrates and how they interact with each other and with the 26S proteasome is hitherto largely unknown. There are five different polyubiquitin receptors in the yeast. Two of them are genuine subunits of the 26S proteasome: the Rpn10 contains a single ubiquitin-interaction motif (UIM) involved in substrate selection, while in the Rpn13 subunit a pleckstrin-like domain (Pru) is responsible for substrate binding. The N-terminal von Willebrand-A domain of Rpn10 and the Pru domain of Rpn13 ensure the binding of these subunits to the 26S proteasome. The other three polyubiquitin receptors of the yeast (the UBA/UBL proteins Rad23, Dsk2 and Ddi1) are extraproteasomal, monomer proteins that bind the

polyubiquitylated substrates by their ubiquitin-associated (UBA) domain and anchor to the proteasome by their ubiquitin-like (UBL) domain.

Earlier studies in yeast suggested that polyubiquitylated proteins collected by the extraproteasomal ubiquitin receptors are targeted to a large docking surface of the proteasome regulatory particle that is formed by the Rpn1/Rpn2 subunits. Recent results, however, suggest that in higher eukaryotes the structure and the mode of action of polyubiquitin receptors may be different to that observed in yeast. Thus, it turned out that the ortholog of Rpn10 in *Drosophila*, subunit p54, is essential, its deletion is lethal in *Drosophila*, while deletion of Rpn10 in yeast has no detectable phenotype. The *Drosophila* p54 subunit has three UIM motifs and a terminal lysine cassette, which is conserved in all higher eukaryotes. Furthermore, it was shown that by developmental-specific differential splicing several protein products are coded by the ortholog of Rpn10 in mice. In these protein products, two UIM motifs appear in different combinations, and the deletion of the embryo specific form is lethal. There are two structurally different essential Rpn10 paralogs in human, one of them, the S5a interacts with the Rad23 extraproteasomal polyubiquitin receptor. From these observations it was shown first in higher eukaryotes and later also in yeast that the binding of the UBA/UBL polyubiquitin receptors to the proteasome is a well orchestrated, controlled process; there are well-defined interactions between the polyubiquitin receptors, and each of the receptors have their own defined substrates.

It is know today, that besides a common regulatory protocol the regulation of the polyubiquitin receptors may differ substantially in higher eukaryotes, depending on the environmental conditions. Contrary to intensive research efforts, there is no detailed, uniform model, which describes the mode of action of the polyubiquitin receptors.

AIMS OF THE STUDY

It was known from our previous *in vitro* studies that in the presence of excess zinc ions, the p54 subunit of the *Drosophila melanogaster* 26S proteasome

dissociates from the proteasome, and this process is accompanied by the disassembly of the 26S proteasome into its subcomplexes and the loss of the catalytic activity. All these changes are fully reversible; removal of the excess zinc ions is followed by the reassembly of the 26S proteasome and the regain of the catalytic activity. In its dissociated form subunit p54 interacts with several proteins involved in protein modification. The reversible dissociation-association of subunit p54 is called shuttling. The *in vivo* relevance of this shuttling was supported by two independent observations:

1. The p54 subunit was present in stochiometric concentration in highly purified 26S proteasomes prepared from early embryos, but only in substoichiometric concentration in purified 26S proteasomes prepared from a mixture of embryos and larvae. This observation suggested that the proteasomal concentration of subunit p54 might vary in a developmentally regulated pattern.
2. Our protein cross-linking experiments suggested that in the presence of ATP subunit p54 is present in two different conformations in the Regulatory Particle. This observation and the interaction of p54 with enzymes involved in protein modifications suggested that postsynthetic modification(s) of the subunit might promote the shuttling of the subunit and influence its function during its extraproteasomal state.

Research plan

We planned the construction of a transgenic *Drosophila* system, which would allow following the *in vivo* fate of subunit p54, to prove or reject the idea of *in vivo* shuttling of subunit p54.

- By the aid of inducible over-expression of Strep-tagged full-length p54 or its different domains, we wanted to chase out the endogenous p54 from the proteasome, which would allow extending the duration of the extraproteasomal state of the subunit to study its postsynthetic modifications or its interactions with other cellular proteins. Transgenic full-length p54 or its N-terminal half,

which carry the vWA domain required for proteasomal interaction, were planned for this purpose. In an alternative approach, we planned to construct a transgenic animal expressing the C-terminal half of p54, lacking the vWA domain required for proteasomal interaction. Induced over-expression of this transgenic protein would model the extraproteasomal state of subunit p54.

- In an independent set of experiments, we planned to measure the changes in the concentration of subunit p54 during the ontogenesis of *Drosophila melanogaster*, and to study the mechanisms, which may regulate its concentration.
- We also planned to analyze the whole spectrum of polyubiquitin receptors functioning in *Drosophila melanogaster*, to map their functional domains and their interactions with each other and with the proteasome.

METHODS

In vitro methods:

- Databases and prediction software applications (NCBI, EBI, FlyBase, IUPred, dsCheck)
- *In vitro* DNA recombination techniques
- Nucleic acid preparation from biological sample (genomic DNA, mRNA, cDNA, plasmid)
- Polymerase chain reaction, semiquantitative reverse transcription-coupled PCR
- Protein preparation from biological samples
- Protein purification
  - Gel-filtration chromatography (FPLC)
  - Affinity chromatographic methods
  - Ion-exchange chromatography (FPLC)
  - Hydrophobic-interaction chromatography (FPLC)
- Separation of proteins by gel electrophoresis (Native-PAGE, denaturing (SDS)-PAGE and 2D-IEF-SDS PAGE)

- Protein identification and visualization:
  - Antibody production and immunoblotting techniques
  - Protein staining by different methods
- Detection of protein-protein interactions and protein modifications:
  - Immunoprecipitation
  - Chemical cross-linking
  - Pull-down experiments with recombinant and endogenous proteins
  - Preparation of samples for mass-spectrometric identification

In vivo methods:

- Establishment of transgenic *Drosophila* stocks by P-element transformation :
  - Induced transgenic protein production in flies
  - Induced gene silencing (RNAi)
- Synchronizing the egg laying of flies
- Analysis of genetic interactions

RESULTS

1) *Drosophila melanogaster* has three polyubiquitin receptors.

Based on sequence- and structural homologies we found that the yeast polyubiquitin receptor orthologs (p54 and Rpn13, Rad23, Dsk2 and Ddi1) are present in *Drosophila melanogaster*, as well. We made cDNA libraries from embryonic and larval total RNA and successfully amplified the cDNAs of the supposed polyubiquitin receptors using specific oligonucleotide primers in PCR reaction. We mapped the functional domains of the GST-tagged recombinant proteins in pull-down experiment and demonstrated that only three out of the five receptors (p54, Rad23 and Dsk2) are active in *Drosophila*. Among them, the proteasome subunit p54 carries three ubiquitin-interacting motifs (UIMs) responsible for the binding of polyubiquitilated proteins, while the Dsk2 and Rad23 carries the ubiquitin-associated domain (UBA) that specifically binds the

substrates. Although we proved that the *Drosophila* Ddi1 and Rpn13 have similar domain structure as it was found in the yeast orthologs, they were unable to bind polyubiquitilated substrates in fruit fly. Using different transgenic stocks (RNA-interference constructs to knock down and protein over-expressing constructs) in *in vivo* studies, we confirmed our *in vitro* result that the p54, Rad23 and Dsk2 are the main polyubiquitin receptors in *Drosophila*.

2) The concentration of the *Drosophila* polyubiquitin receptors changes in a developmentally regulated fashion.

Total protein extracts were prepared from animals in different developmental stages, and the subunit compositions (including the p54) of the 26S proteasome were analyzed by immunoblotting technique. We found that the concentration of the p54 polyubiquitin receptor subunit, relative to those of other proteasome subunits falls suddenly at the end of embryogenesis, remains low throughout the larval stages, starts to increase again in the late third instar larvae and remains high in the pupae, adults and embryos. A similar developmentally regulated fluctuation was observed in the concentrations of the Rad23 and Dsk2 extraproteasomal polyubiquitin receptors. Surprisingly, in contrast to the serious reduction in the concentrations of the polyubiquitin receptors, there was no detectable accumulation of the polyubiquitylated proteins during the early larval stages. We suppose that during the larval stages the catalytic activity of the proteasome is muted, although, we have only speculations on the biological relevance of this phenomenon.

3) **Polyubiquitin receptors are selectively degraded during the larval stages.**

We demonstrated that the level of the mRNAs encoding the p54, Rad23 and Dsk2 polyubiquitin receptors remained constant during the ontogenesis of *Drosophila.* It suggested that their depletion in the early larval stages is not transcriptionally regulated. We found that the polyubiquitin receptors are proteolytically degraded by a specific protease. We purified the enzyme till homogeneity and identified it as a serine-protease by mass spectrometry. Although

the *in vitro* and *in vivo* examination of the serine-protease is still in progress, we proved that the enzyme is essential, it is active throughout the larval stages, and it is inhibited (transcriptionally and by a selective inhibitor) at the end of the larval cycle.

Although we don't know the relevance of this regulation, we suppose that the irreversibly misfolded proteins that are proteasome substrates in non-larval stages, are degraded by larvae-specific serine-proteases in larval stages. Thus, the activity of the proteasome has to be limited in larvae. The elimination of the polyubiquitin receptors by a serine-protease is the simplest and most economical way of proteasome inactivation.

4) Only the C-terminal half of p54 is degraded during the larval stages.

We found that during the degradation of p54 the N-terminal domain of the protein remains intact. We predicted and experimentally confirmed that the C-terminal part of p54 is intrinsically unstructured. It turned out that the serine-protease identified as polyubiquitin receptor-hydrolyzing enzyme selectively degrades the unstructured region within p54. Because Rad23 and Dsk2 also contain extended unstructured regions, we suppose that the selectivity of the serine-protease is related to this structural feature of the polyubiquitin receptors.

5) Transgenic p54 and its N-terminal half incorporated into the proteasome.

To follow the *in vivo* fate of subunit p54, transgenic lines encoding the Strep-tagged N-terminal half (NTH) or full-length p54 subunits have been established in the inducible Gal4-UAS system. We demonstrated that the whole-body expression of the full-length subunit or its NTH did not produce any detectable phenotypic changes and these vWA domain bearing transgenic products were quantitatively incorporated into the 26S proteasome. Our original goal to chase out the endogenous p54 failed due to the low expression level of transgenic proteins. According to our indirect results we suppose that the coordinated regulation of the genes encoding proteasomal subunits limited the over-expression of the transgenes.

6) <u>The C-terminal half of p54 is accumulated extraproteasomally, it is ubiquitylated in its terminal lysine residues and caused third instar larval lethality.</u>

To mimic the extraproteasomal state of p54, transgenic stocks encoding the C-terminal half of p54 (p54-CTH) has been established. As expected, gel-filtration chromatography revealed that the ectopically over-expressed transgenic p54-CTH lacking the vWA domain was not incorporated into the 26S proteasome, accumulated extraproteasomally and caused third instar larval lethality. We affinity purified the Srep-tagged version of p54-CTH and its covalently modified derivatives from larvae, further fractionated them on 2D-IEF-SDS-PAGE and analyzed by mass spectrometry. We proved that the p54-CTH is extraproteasomally ubiquitylated. Our *in vivo* studies revealed that the p54-CTH is ubiquitylated in its terminally localized conserved (in higher eukaryotes) lysines, and this ubiquitylation is definitely not a degradation signal to the proteasome. We suppose that the C-terminal conserved lysines of p54 are mono-and/or multiubiquitylated, and this postsynthetic modification is responsible for the shuttling and structural integrity of the protein.

7) <u>The serine-protease mediated developmental-stage-specific regulation of polyubiquitin receptors is inhibited by the p54-CTH.</u>

We found that the polyubiquitin receptors accumulate in earlier larval stages in p54-CTH over-expressing transgenic animals than in wild-type controls. We proved that the extraproteasomally accumulated and multiubiquitylated transgenic p54-CTH transcriptionally limited the larval expression of the selective serine-protease responsible for degradation of polyubiquitin receptors. This observation suggests that the serine-protease and the post synthetically modified p54-CTH mutually regulate each other in order to fine-tune the ubiquitin-proteasome system during the ontogenesis.

8) p54-CTH inhibits the proteasomal degradation of polyubiquitylated protein.

Surprisingly, contrary to the premature accumulation of the polyubiquitin receptors following the over-expression of p54-CTH, there was a significant accumulation of polyubiquitylated proteins in early larval stages. How can we interpret this observation? We found that the extraproteasomally-localized p54-CTH, which carries three UIMs, efficiently binds not only the p54-specific polyubiquitylated proteins, but also the Dsk2 extraproteasomal polyubiquitin receptor. In this way, the p54-specific substrates and the Dsk2 are entrapped, due to the lack of the vWA domain of p54-CTH. Thus, their transport to the proteasome is completely hindered, resulting in the accumulation of the polyubiquitylated proteins. The disturbance in the balance of polyubiquitylated substrate degradation causes the death of p54-CTH over-expressing transgenic animals.

9) The *Drosophila* Dsk2 binds to the proteasome through the p54 subunit.

Our *in vitro* pull-down experiments revealed that the Dsk2 UBL efficiently binds to the third UIM of p54 polyubiquitin receptor. Furthermore we proved that proteasomes lacking the p54 subunit (prepared from p54-null- or p54-silenced *Drosophila* mutants) were unable to bind to the Dsk2 protein, although, the binding was efficient in the case of p54-containing wild-type proteasomes. Using polyubiquitin receptor over-expressing and/or silencing transgenic lines, we found strong genetic interaction between p54 and Dsk2. According to our *in vitro* and *in vivo* results, contrary to the yeast model in which Dsk2 binds to the proteasome via the Rpn1/Rpn2 surface, the *Drosophila* Dsk2 interacts with the proteasome through the p54 shuttling factor.

CONCLUSIONS

The cellular concentration of the three main polyubiquitin receptors of *Drosophila melanogaster* (the proteasomal subunit p54 and the extraproteasomal Dsk2 and Rad23 proteins) is developmentally regulated. The concentration of the receptors is very high during the embryonic-, pupal- and adult stages, ensuring high activity of the ubiquitin-proteasome system. We suppose that during these developmental stages a specific ubiquitin-ligase multiubiquitylates the terminal lysines of subunit p54, which induces a conformational change in the subunit required for its dissociation from the proteasome. In addition, the multiubiquitylation may stabilize the subunit during its dissociated state, protecting it against the proteolytic action of cytosolic proteases. As the dissociation-association equilibrium of subunit p54 is shifted towards the reassociation, the duration of the extraproteasomal state of the subunit is very short. In the dissociated state of subunit p54 its UIM1 and UIM2 motifs recruit and bind a specific set of polyubiquitylated proteins, which are processed for proteolysis exclusively by this polyubiquitin receptor. The third UIM motif of p54 recruits and binds the UBL domain of Dsk2 charged with its own polyubiquitylated substrates. In its uncharged state the UBL domain of Dsk2 is masked by an intramolecular interaction with the UBA domain of the same Dsk2 receptor molecule, preventing its interaction with subunit p54. As the UBA domain of Dsk2 has much higher affinity for polyubiquitylated proteins than the for its own UBL domain, in the presence of a suitable polyubiquitylated protein (belonging to the group of specific substrates processed by Dsk2) the UBL domain is unmasked, ensuring its association with the UIM3 motif of p54. This mechanism ensures that p54 will recruit and bind only substrate-charged Dsk2 receptors. Following the deubiquitylation of p54, the subunit will reassociate with the proteasome. The reassociation, which is supported by chaperon proteins, presents the polyubiquitylated proteins for proteasomal degradation. The shuttling of p54 will

coordinately present two different sets of polyubiquitylated proteins for degradation.

During the larval stages, due to the almost complete cessation of mitotic activity, the generation of polyubiquitylated proteins drops to a low level, that is accompanied by a decline of proteasomal activity. This decline is ensured by a proteolytic degradation of the polyubiquitin receptors. During the larval stages an essential serine-protease emerges, which can selectively recognize and degrade the unstructured regions of free- and proteasome-bound polyubiquitin receptors, leading to the inactivation of the ubiquitin-proteasome system. We suppose that during the larval stages, due to the hypoxic life conditions, a large amount of irreversibly denatured protein is produced, which are removed by a specific serine-protease capable of more efficiently degrading these damaged proteins than the proteasome. At the end of the larval developmental phase, this selective proteolytic activity is abolished by the appearance of specific inhibitor. This results in the accumulation of polyubiquitin receptors, as the concentration of the polyubiquitin receptor-specific mRNAs is constant during the development. The accumulated p54 in its multiubiquitylated form inhibits the transcription of the gene encoding this specific serine-protease, accelerating the reactivation of the ubiquitin-proteasome system.

The biological relevance of this developmental regulation is not known in all details. We have to emphasize that the very high mitotic activity during the embryonic stage, the histolysis of the larval structures during the pupal stages as well as the high reproductive activity during the adult stage requires high activity of the ubiquitin-proteasome system, which is ensured by high polyubiquitin-receptor concentration.

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FULL PAPERS

⇒ **Lipinszki, Z**., Kiss, P., Pal, M., Deak, P., Szabo, A., Hunyadi-Gulyas, E., Klement, E., Medzihradszky, K. F. and Udvardy, A. (2009) Developmental-stage-specific regulation of the polyubiquitin receptors in Drosophila melanogaster. Journal of Cell Science. 122, 3083-3092
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⇒ Articles related to the Ph.D. thesis