Genetic and biochemical studies on the gene encoding proteasomal subunit S5a/Rpn10/p54 in Drosophila melanogaster

Ph.D. Thesis

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Introduction

The dynamic turnover of cellular proteins is maintained by a regulated balance of protein synthesis and degradation. In the selective degradation of intracellular proteins, decisive roles are played by an enzyme cascade and a large proteolytic complex, the 26S proteasome. The enzyme cascade is able to recognise the different "degradation signals" present in short-lived proteins and to modify these proteins by the covalent attachment of a multiubiquitin chain. The same enzyme cascade is responsible for the multiubiquitination of damaged or misfolded proteins. Multiubiquitinated proteins are recognised, bound and degraded by the 26S proteasome.

This large proteolytic complex is composed of two distinct subcomplexes: the regulatory complex (RC) and the catalytic core. The 20S proteasome, the catalytic core, is a barrel-shaped multicatalytic protease. Three nanocompartments are located inside the 20S proteasome, connected to each other by a narrow central channel. The central nanocompartment contains the catalytic centres, while the functions of the peripherial nanocompartments are still unknown. In consequence of the narrowness of the central channel and the gated nature of its orifice, the catalytic centres of the 20S proteasome are inaccessible for folded proteins.

Protein unfolding is probably one of the most important functions of RCs. The chaperone-like activity of the RC may be responsible for protein unfolding. Unfolding of the substrate proteins is most probably an ATP-dependent step, and the six ATPase subunits present in the RC may perform the ATP hydrolysis required in this process. Opening of the central channel of the catalytic core is performed by one of the ATPase subunits of the RC, suggesting that channel opening is also an energy-dependent function. Although no direct experimental evidence is available, it is reasonable to suppose that the feeding of unfolded proteins into the gated central channel of the 20S proteasome is also an energy-dependent function of the RC.

As the 20S proteasome is a non-specific protease, the selectivity of the 26S proteasome towards multiubiquitinated proteins should be ensured by the RC. This assumption is supported by the observation that S5a/Rpn10/p54 (human/yeast/*Drosophila*) is one of the RC subunits of the 26S proteasome, which can recognise and bind multiubiquitin chains *in vitro*.

The role of S5a/Rpn10/p54 in substrate recognition is debated in view of the observation that deletion of this subunit in yeast is not lethal, and has only a mild phenotype. Furthermore, it was recently reported, that *in vitro* studies revealed selective cross-linking of a reactive multiubiquitin chain to S6'/Rpt5/p50, an ATPase subunit of the RC. Deletion of S5a/Rpn10/p54 in the haploid moss *Physcomitrella patens*, however, causes developmental arrest, and the polyubiquitin-binding site of the fission yeast homologue of S5a/Rpn10/p54 is essential when the S14/Rpn12/p30 subunit is compromised.

In order to gain an insight into the function of this RC subunit in higher eukaryotes, we have generated a *Drosophila* mutant by deleting the single copy gene of subunit p54 (this gene is annotated in GadFly as *pros54*) and analysed the molecular changes and phenotypic effects of the deletion. The results summarized in my Thesis were published in the Journal of Cell Science (Szlanka *et. al.*, 2003., *J. Cell Sci.*; 116, 1023-33).

Methods

Classical genetic works:

- **Genetic crosses with wild type and different mutant stocks of *Drosophila melanogaster*,**
- **P-element mobilization,**
- **P-element-mediated germ line transformation,**
- **Lethal phase analysis,**
- **Phasecontrast-microscopic analysis of orcein-stained chromosome preparations**

Molecular biological methods:

- **Native and SDS Protein gelelektrophoresis,**
- **Immunoblot,**
- **Southern blot,**
- **PCR,**
- **Molecular cloning,**
- **DNA Sequencing**

Results and discussion

To obtain a null allele of *pros54*, we screened by Southern blotting a series of *P*-element insertions from a large-scale insertional mutagenesis experiment on the third chromosome. The *P-lacW* insert in line 0554/18 was found near the 3' end of *pros54*. Started from this line, we isolated a series of chromosomal deletions generated by *P*-element-induced male recombination. Genomic DNAs were then screened for deletions by PCR analysis. Sequencing of the PCR products identified a line in which the

deletion eliminated more than 90% of the coding region of *pros54*. This 2095 bp deletion also eliminated two other genes located between *pros54* and the P-element. In the deletion homozygotes the functions of these two genes (*CG7181* and *Vha M9.7-2*) were rescued by a plasmid construct carrying the genomic fragment of the two genes. This genetic combination, which lacked only the *pros54* gene was named *Δp54*.

Western blot analysis revealed that *Δp54* animals did not produce any detectable p54 protein, and therefore these animals represent the null phenotype of *pros54*. *Δp54* mutant animals display polyphasic lethality during all the larval and pupal phases. The examination of mitotic cells in squashed preparations of the central nervous system from *Δp54* third instar larvae revealed multiple mitotic defects. Both the mitotic index and the metaphase:anaphase ratio are increased two times as compared to that in the wild-type. Moreover, the frequency of some abnormal mitotic figures (overcondensed chromosomes, prematurely separated sister-chromatids, circular mitotic figures, aneuploid and polyploid figures) is also high in the mutant. These features arose as the consequence of mitotic arrest and indicate that *Δp54* cells can enter mitosis, but their progression through and exit from mitosis is delayed or blocked for some time.

As S5a/Rpn10/p54 is considered to be responsible for the substrate selection of the 26S proteasome, the observation that deletion of the gene encoding this subunit does not influence the viability of yeast cells was unexpected. This suggested a hypothesis that substrate recognition is a more complicated process probably involving several different, partially overlapping mechanisms.

The finding that the degradation of certain proteasome substrates is impaired in the yeast Df(S5a/Rpn10/p54) mutant, on the other hand supports the notion that S5a/Rpn10/p54 functions alone as a multiubiquitin receptor for certain substrate proteins, and no other protein is involved in this function. The mild phenotype of this mutant, however, suggests that the number of the multiubiquitinated proteins recognised and targeted exclusively by this RC subunit in the yeast is limited. The lethality of *Δp54* indicates that in *Drosophila* either the

number of multiubiquitinated proteins processed exclusively by the S5a/Rpn10/p54 subunit is much larger, or during the pupal developmental phase a limited number of key substrate proteins have to be processed exclusively by this RC subunit, and insufficient degradation of these proteins can block the developmental program resulting in lethality. The severe mitotic defects observed in the larval brain of the mutant suggest that proteins involved in the cell cycle regulation may belong to this specific class of substrate proteins.

The developmental profile of subunit p54 was analysed by biochemical methods. It was demonstrated previously that the 26S proteasome (and its subunit p54) is present in very high concentration in wild type *Drosophila* embryos. Western blot analysis revealed that the concentration of this maternally deposited p54 (together with that of the other subunits) declines gradually during the larval stages and increases sharply during the first 4 hours of pupal development due to zygotic gene expression. This is in good accordance with our finding that all of the animals, which reached the pupal phase died at the beginning of this developmental stage. So the viability of *Δp54* embryos and larvae is due to a large pool of maternally stored 26S proteasome in the embryos, which becomes only gradually depleted during the larval stage. This does not exclude the possibility that the S5a/Rpn10/p54 subunit is essential for the appropriate functioning of the proteasome in every cell, through all phases of the development, or that, similarly to the yeast cells, it is generally dispensable, but essential only in certain phases of the development. The polyphasic larval-pupal lethality of the *Δp54* mutant, however, suggests that, as soon as the maternally stored wild-type 26S proteasome depot is depleted, mutant proteasomes, even in large excess, can not rescue the lethality.

Immunoblot analysis with an anti-ubiquitin antibody showed that there is a shift in the proportion of highly multiubiquitinated proteins in the pupae of the deletion mutant. Fractionation of freshly prepared protein extracts from mutant pupae on a native polyacrylamide gel revealed that, opposite to yeast, the lack of subunit p54 does not destabilizes the RC and does not interfere with the assembly of the RC

and the catalytic core in *Drosophila*. The undisturbed assembly of the RC and the catalytic core, and the lack of gross structural disintegration of the 26S proteasome in the *Δp54* animals, strongly suggest that the pupal lethality of the mutant is due to the impairment of some specific function of the proteasome in consequence of the lack of subunit p54.

By Western blotting, we found a huge and coordinated accumulation of proteasomal subunits in the *Δp54* animals. This suggests that a feedback circuit regulating the coordinated expression of proteasomal genes also exists in higher eukaryotes. In the yeast, RPN4 was identified as a transcription factor involved in the coordinated regulation of genes encoding proteasomal subunits. The observations that RPN4 can coordinately enhance the expression of proteasomal genes, and that at the same time it is degraded by the proteasome, led to the supposition of a feedback circuit in yeast. In higher eukaryotes, the fully coordinated regulation of genes encoding proteasomal subunits has not been demonstrated before. The extreme accumulation of proteasomal subunits in the *Δp54* animals lends strong support to the existence of such a feedback circuit. The large mass of the proteasomal subunits in this mutant is present in the form of fully assembled proteasomal particles. The lack of free subunits and/or partially assembled proteasomal complexes is a direct indication of a fully coordinated regulation of the expression of all proteasomal subunits. A transcription factor homologous to the yeast RPN4 has not hitherto been identified in higher eukaryotes. The extreme and coordinated overexpression of the proteasomal subunits in *Δp54* mutant, however, indicates that a transcription factor(s) capable of coordinately regulating the expression of proteasomal genes must also function in higher eukaryotes.

Publication Used for the Thesis

Szlanka, T.; Haracska, L.; Kiss, I.; Deák, P.; Kurucz, E.; Ando, I.; Virágh, E.; Udvardy, A. (2003) Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in Drosophila melanogaster. *J. Cell Sci.*; 116, 1023-33.

Other Publications

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