

PhD THESES

**MOLECULAR GENETIC AND FUNCTIONAL ANALYSIS OF THE RPT1
SUBUNIT OF THE *DROSOPHILA MELANOGASTER* 26S PROTEASOME**

ÁRON SZABÓ



Supervisor: Andor Udvardy, Ph.D., D.Sc.

Biological Research Centre of the Hungarian Academy of Sciences
Institute of Biochemistry
Intracellular Protein Degradation Group

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Introduction

The ubiquitin-proteasome system (UPS) is responsible for the regulation of a large variety of biological processes. The controlled intracellular proteolysis is perhaps the most thoroughly studied function of this system. The ubiquitination enzyme cascade is the first main component of the UPS. The three enzymes involved in this cascade fulfill two critical functions: i./ recognition of specific cellular proteins intended for intracellular proteolysis, and ii./ via the covalent attachment of ubiquitin chains to the selected proteins, their marking for intracellular proteolysis. The 26S proteasome, the second main component of the UPS, is a large multiprotein complex which is responsible for the recognition, binding and proteolytic degradation of multiubiquitinated proteins. The 26S proteasome is assembled in an ATP-dependent reaction from two main subcomplexes: the catalytic particle (CP or 20S proteasome) and the regulatory particles (RP). The 20S proteasome is a barrel-shaped particle composed of α - and β -type subunits that form seven-membered rings stacked together in the $\alpha_7\beta_7\beta_7\alpha_7$ configuration. Three nanocompartments are located inside this particle, connected to each other by a narrow central channel. The site of protein degradation is the central nanocompartment; the functions of the two peripheral nanocompartments are not fully known.

The RPs are attached to one or to both bases of the CP and in *Drosophila melanogaster* are built up from 18 different subunits. Among them, 6 subunits belong to the AAA-type ATPases; all the others are structurally diverse. During conventional chromatographic purification procedures, the RP may be split into base and lid subcomplexes as a result of the artificially high ionic strength. The ATPase subunits, together with three non-ATPase subunits, form the base subcomplex. The lid subcomplex is composed entirely of non-ATPase subunits. The RPs perform several critical functions of the 26S proteasome, ensuring the selectivity of the 26S proteasome for multiubiquitinated proteins, unfolding the substrate proteins by their chaperone-like activity, opening the gated channel of the CP, reprocessing the ubiquitin residues of the substrate proteins and feeding the substrate into the CP. The contribution of the individual RP subunits to the different steps of the proteolytic process is far from clear. Subunit Rpn10/p54 in cooperation with soluble, non-proteasomal proteins, is responsible for the selective recognition and binding of multiubiquitinated proteins. A subunit of the lid subcomplex Rpn11/p37B, which

contains a novel Zn^{2+} -metalloprotease domain, is responsible for reprocessing the ubiquitin moieties of the multiubiquitinated substrate proteins. Rpn1 and Rpn2, two non-ATPase subunits of the base, link the lid and base subcomplexes.

Several steps of the proteolytic cycle are ATP-dependent. Besides the assembly of the 26S proteasome from its subcomplexes, ATP is required for substrate unfolding, opening the gated channel of the CP, and most probably feeding the substrate proteins into the central channel of the CP. Six ATPase subunits of the RP, forming a heterohexameric ring, mediate all the ATP-dependent reactions. The ATPase ring stacks to the base of the external α -rings of the 20S proteasome, this configuration ensuring optimum access for ATPase subunit Rpt2 to open the gated channel of the CP by displacing N-terminal segments of α -type subunits involved in the gating. Unfolding of the substrate protein is probably performed by concerted action of the ATPase ring. Mutagenesis studies in yeast indicate that the individual ATPase subunits execute distinctly different functions, despite the high level of sequence similarity of the ATPase subunits. Our knowledge concerning the functions of the different ATPase subunits in higher eukaryotes is rather limited.

Specific aims

The unique features of proteasomal ATPases, especially in higher eukaryotes, are still hardly known. The collection of P-element insertional *Drosophila melanogaster* mutants is now widely available. Our aim was to characterize the ATPase subunits of the regulatory complex, starting with Rpt1, through analysis of *Drosophila* mutant lines. We investigated in a hypomorphic *Rpt1* mutant line:

- the nature of the mutation
- the structural features of the proteasome
- the accumulation of multiubiquitylated and substrate proteins
- phosphorylation of Rpt1 and its localization onto chromatin
- the occurrence of Rad23B protein
- mitotic features and genetic interactions of the mutation.

We suppose that the *Rpt1* mutants will elucidate the possible role of this subunit in proteasomal assembly, regulation, substrate recognition and its influence on transcription.

Methods

- Recombinant DNA technology
- Polymerase Chain Reaction (PCR)
- Inverse PCR
- Semiquantitative reverse transcription coupled PCR (RT-PCR)
- Real-time PCR
- DNA sequencing
- P-element remobilization
- P-element transformation
- Native gel electrophoresis
- Western blot
- Two-dimensional gel electrophoresis
- Gel filtration chromatography
- Phosphatase treatment
- Salivatory gland polytene chromosome preparation and immunostaining
- Neuroblast preparation and cytological characterization

Results

Four P-element insertion mutants (*Rpt1*⁰⁵⁶⁴³, *Rpt1*^{k1110}, *Rpt1*^{EP(2)2153} and *Rpt1*^{l(2)SH0675}) and one EMS induced mutant (*Rpt1*^{43Ed-1}) affecting the Rpt1/p48B RC ATPase subunit of the *Drosophila* 26S proteasome were obtained. The sites of P-element insertions were determined by inverse PCR. In three strains the site of P-element insertion was exactly the same: between bp 31

and 32 in the 5'-non-translated region (UTR) of the gene. In *Rpt1*^{EP(2)2153} the site of P-element insertion was between bp 22. and 23. of the 5'-UTR. Animals homozygous for *Rpt1*⁰⁵⁶⁴³, *Rpt1*^{k1110} and *Rpt1*^{EP(2)2153} P-element insertions showed 2nd instar larval (L2) lethality, while the lethal phase of the homozygous *Rpt1*^{l(2)SH0675} was shifted to the pupal stage. Homozygous *Rpt1*^{43Ed-1} was also L2 stage lethal. Sequence of this allele revealed a glycine→aspartic acid change at the 382. position of the Rpt1 protein. In complementation tests the Rpt1 mutants did not show complementation except *Rpt1*^{43Ed-1} which showed pupal lethality instead of L2 lethality as transheterozygote with deletions uncovering the *Rpt1* region.

As the *Rpt1/p48B* gene resides inside the long second intron of an annotated, but uncharacterized gene (CG17985), the lethal phenotype of the homozygous P-element insertions may be a consequence of the disruption of the expressions of both genes. In an attempt to assign the lethal phenotype to the *Rpt1/p48B* gene, remobilization of the *Rpt1*^{k11110} P-element insertion was induced. In the excision mutants various P-element sequences of different length remained at the site of P-element insertion. In recessive L2 lethals 250-1000 bp P-element sequence remained while in the pupal lethals only 24-160 bp .Thus longer insertions induce earlier lethal phase.

Transgenic rescue experiments were performed to analyze the role of the damage to the *Rpt1/p48B* gene in the generation of the lethality. In these rescue experiments, the enhancer-promoter region of the *Ubi63E* gene was ligated to the complete coding sequence of the *Rpt1/p48B* gene, and this construct was inserted into the P{pCaspR4} P-element transformation vector. Transgenic stock was generated and was crossed to the *Rpt1*^{L15} larval lethal and *Rpt1*^{P1} pupal lethal mutants and also to two of the original P-element insertion stocks (*Rpt1*⁰⁵⁶⁴³ and *Rpt1*^{k11110}). The flies that were homozygous for the *Rpt1*^{P1} mutation and carried one copy of the rescue construct were fully viable and fertile, indicating that the pupal lethality of this mutation is due to the damage to the *Rpt1/p48B* gene. The transgene carrying the *Rpt1/p48B* gene, however, could only rescue the second instar larval lethality of *Rpt1*^{L15}, *Rpt1*⁰⁵⁶⁴³ and *Rpt1*^{k11110} until pupal stage, suggesting that the P-element insertion damaged the expression and/or the splicing of the uncharacterized gene CG17985, and this gene is essential.

We analysed the relative Rpt1 mRNA content of partially rescued *Rpt1*^{L15} mutant and not-rescued *Rpt1*^{l(2)SH0675} mutant pupae in order to determine to what extent we could rescue the failure of the *Rpt1* gene in the *Rpt1*^{L15} mutant and what was the reason for the pupal lethal phenotype of *Rpt1*^{l(2)SH0675}. We found with RT-PCR that the level of *Rpt1* mRNA in the rescued

Rpt1^{L15} mutant was a little higher, while in the *Rpt1*^{l(2)SH0675} mutant about equal to the *Rpt1* mRNA content of the wild-type. Thus the pupal lethality in both cases was assigned mostly to the mutation of another gene, probably of *CG17985*. Further RT-PCR-s showed that the *CG17985* mRNA level is about the same in the *Rpt1*^{P1} mutant as in the wild-type.

Sequence analysis of the *Rpt1/p48B* promoter explained the events leading to lethality in P-element excision mutants. The *Rpt1/p48B* gene, like the majority of the *Drosophila* genes, has a TATA-less promoter. In general, two essential promoter sequences are involved in the transcriptional regulation of the *Rpt1* gene: the initiator element (at -2-+4 bp) and the downstream promoter element (DPE at +28-32 bp). The distance between these elements is critical for the transcriptional regulation of the gene. The P-elements in the analyzed mutants were inserted between the initiator and the DPE element. The P-element insertions and their derivatives displace the initiator element and the DPE. It was reasonable to suppose that a non-optimum initiator-DPE distance impairs the expression of the *Rpt1/p48B* gene, leading to pupal or larval lethality, depending on the extent to which the two critical regulatory sequences are displaced.

To test this assumption, total RNA was extracted from wild-type and *Rpt1*^{P1} mutant pupae and the concentration of the *Rpt1/p48B* mRNA was estimated by a semiquantitative RT-PCR analysis. Even with this semiquantitative test, the concentration of the *Rpt1/p48B* mRNA seemed to be greatly reduced in the mutant pupae. To make this measurement quantitative, the concentrations of the *Rpt1/p48B* mRNA were measured in the wild-type and *Rpt1*^{P1} mutant pupae by means of an RT-real-time PCR technique. Normalized to 18S rRNA, the concentration of the *Rpt1/p48B* mRNA was 30-fold lower in the P1 mutant pupae as compared with the wild-type pupae. As an internal control, the concentrations of *rosy* mRNA were also measured in the same RNA preparations under the same conditions. There was no significant difference in the concentration of the *rosy* mRNA in the wild-type and the *Rpt1*^{P1} mutant pupal RNA. The pupal lethal P1 mutation is therefore a hypomorphic mutation of the *Rpt1/p48B* gene.

The *Rpt1/p48B* protein contents of the wild-type and the *Rpt1*^{P1} mutant pupae were estimated by immunoblot analysis. In this experiment, total protein extracts were prepared from wild-type and *Rpt1*^{P1} mutant pupae, and the protein contents of the extracts were normalized to the concentrations of the glycogen phosphorylase household protein estimated by immunoblot staining. Equal amounts of wild-type and P1 pupal protein were loaded for SDS-PAGE and

immunoblotted with an anti-Rpt1/p48B antibody. The Rpt1/p48B protein content of the P1 mutant was much less than that of the wild-type pupae. The polyclonal anti-Rpt1/p48B antibody recognized a closely-spaced triplet both in the wild-type and in the *Rpt1^{P1}* mutant pupal protein extracts. While the electrophoretic mobilities of the individual bands of the triplet were indistinguishable in the wild-type and the mutant pupal protein extracts, the relative intensities of the bands differed significantly. As the human orthologue of the *Drosophila* Rpt1/p48B subunit has been shown to be phosphorylated, we attempted to prove that the higher molecular weight bands recognized by the polyclonal antibody correspond to different phosphorylated forms of the protein. For this purpose, total protein extracts of wild-type and *Rpt1^{P1}* mutant pupae were separated by 2D IEF-SDS-PAGE and analyzed by immunoblotting with the polyclonal anti-Rpt1/p48B antibody. The polyclonal antibody recognizes at least 6 distinct spots which differ in isoelectric point, strongly suggesting that the Rpt1/p48B protein is multiphosphorylated in the pupae. The 2D gel electrophoresis results confirmed the observation obtained from 1D SDS-PAGE that the relative proportion of the multiple-phosphorylated forms is higher in the mutant pupae as compared with the wild-type. To determine the developmental profile of the multiphosphorylation pattern of Rpt1/p48B protein, total protein extracts of wild-type embryos, pupae and flies were analyzed by 2D IEF-SDS-PAGE. The phosphorylation pattern of Rpt1/p48B protein in pupae and flies was indistinguishable, in embryos, however, only mono-, di-, tri- and tetraphosphorylated Rpt1/p48B forms were present, the most highly phosphorylated forms present in pupae and flies were not detectable.

To prove unequivocally that the different isoforms of Rpt1/p48B detected by 2D IEF-SDS-PAGE correspond to different multiphosphorylated forms of the subunit dephosphorylation by potato acid phosphatase was attempted. This phosphatase efficiently dephosphorylated two ATPase subunits of the human RP. To overcome the potential steric hydrance of the ATPase ring by the CP the 26S proteasomes were disassembled into free RP and CP by ATP removal. For this purpose a total embryonic protein extract was fractionated on a Sephadex G25 spin column equilibrated with the reaction buffer of the acid phosphatase (50 mM PIPES pH=5.8, 1 mM DTT). Proteins which appeared in the exclusion volume were incubated with or without 1 U/ml potato acid phosphatase for 1 hour at 30 °C, fractionated on 2D IEF-SDS-PAGE and analyzed by immunoblotting. Following dephosphorylation the multiplicity of the Rpt1/p48B isoforms with different isoelectric points disappeared and only a single spot was detectable with the antibody.

Immunoblotting analysis of total protein extracts prepared from wild-type and *Rpt1^{P1}* mutant pupae with monoclonal antibodies specific for lid or other base subunits of the RP revealed moderate increases in the relative concentrations of the other RP subunits in the mutant pupae.

The Rpt1/p48B subunit is a member of the heterohexameric ATPase ring of the RP which provides a binding surface for the CP during the assembly of the 26S proteasome. Native polyacrylamide gel electrophoresis was used to test how the severe decrease in the Rpt1/p48B protein affects the structure of the 26S proteasome. Total protein extracts prepared from wild-type and *Rpt1^{P1}* mutant pupae were fractionated on 3.8% native polyacrylamide gel, blotted onto a PVDF membrane and developed with different RP- or CP-specific monoclonal antibodies. In the wild-type pupal extracts, the characteristic 26S proteasome pattern was detected, the doubly-capped and the singly-capped 26S proteasome bands reacted with base-, lid- and CP-specific antibodies, and only a small quantity of free CP was present, indicating that the majority of the 20S proteasome particles were assembled into 26S forms. A completely different pattern was observed in the mutant pupal protein extract. Here, the characteristic doublet of the 26S proteasome was completely missing; a strong smeary band reacting with base-, lid- and CP-specific antibodies was present, while the majority of the CP appeared as free particles. This pattern clearly indicates severe damage to the 26S proteasome assembly. The limited concentration of the Rpt1/p48B protein present in the mutant is sufficient only for the assembly of a small amount of the intact ATPase ring, and thus the probability of detecting intact 26S proteasomes is negligible. Although a small amount of 20S proteasome is able to bind to the incomplete RPs assembled without the Rpt1/p48B subunit, the 26S proteasome particles formed in this way will have an irregular shape, resulting in the formation of a large mass of smeary band, which reacts with base-, lid- and CP-specific antibodies alike.

As a consequence of the reduced concentration of intact 26S proteasome particles, an accumulation of highly multiubiquitinated proteins can be detected in the P1 mutant pupae.

RP subunits, and especially those belonging to the base subcomplex, are thought to have essential functions inside the chromatin in the regulation of transcription. By immunofluorescent staining, we analyzed the distribution of the Rpt1/p48B protein in the polythene chromosomes of the salivary gland of wild-type and *Rpt1^{P1}* mutant larvae. In the wild-type larvae, strong Rpt1/p48B immunostaining was detected over the polythene chromosomes with a characteristic

banding pattern. Double immunostaining with an anti-RNA polymerase II specific monoclonal antibody revealed that the staining patterns and staining intensities obtained with these antibodies were very similar over the majority of the bands. Inside puffed regions of the polytene chromosomes, however, completely different picture appeared. In consequence of the strong decondensation of the chromatin, the RNA polymerase II staining produced only a faint, narrow band, which is in sharp contrast with the huge accumulation of the Rpt1/p48B protein over the whole area of the puffed regions. The faint, nonspecific staining with the anti-Rpt1/p48B antibody in the *Rpt1^{P1}* mutant larvae is in sharp contrast with the wild-type staining pattern. The greatly reduced Rpt1/p48B protein concentration in the mutant resulted in an almost complete depletion of this ATPase protein from the chromatin. The lack of the Rpt1/p48B protein in the polytene chromosomes did not influence the distribution of the RNA polymerase II.

The severe structural and functional disturbance of the 26S proteasome in the *Rpt1^{P1}* mutants induced unexpected changes in the cellular concentration of a proteasome-interacting protein. In *Drosophila*, the Rad23 protein, one of the extraproteasomal ubiquitin receptors, is present in two splice variants. Immunoblot analysis with a polyclonal anti-Rad23 antibody revealed that in the wild-type *Drosophila* pupae only the Rad23A protein is present in significant concentration, Rad23B being undetectable. The sequence homology between the A and B splice variants of Rad23 extends over a long segment, and accordingly a polyclonal antibody should recognize both variants. The lack of reaction indicates that the concentration of Rad23B protein is very low in the wild-type pupae, below the detection limit of our antibody. In the *Rpt1^{P1}* mutant pupae, however, a substantial amount of Rad23B protein can be detected, though Rad23A is the predominant form. The presence of Rad23B in the mutant pupae may be a consequence of a change in the differential splicing of the Rad23 gene. Our RT-coupled real time PCR analysis, however, suggests that the accumulation of the Rad23B protein is more probably due to impaired proteasomal degradation of this isoform. Quantitative real-time PCR analysis indicated that the concentrations of the Rad23B mRNA in the wild-type and the *Rpt1^{P1}* mutant pupae were practically identical.

The activity of the 26S proteasome is essential for normal cell cycle progression. To determine the role or the contribution of subunit Rpt1/p48B to the overall function of the proteasome in the cell cycle, we analyzed the morphology of mitotic cells in neuroblast preparations from *Rpt1^{P1}* mutant larvae. It was quite surprising that, in contrast with the severe

shortage of the functional Rpt1/p48B protein, mitotic defects did not develop in the *Rpt1^{P1}* mutant. In *Saccharomyces cerevisiae* the mutation of the Rpt1 gene results in serious mitotic block and the accumulation of the mitotic cyclins. In agreement with the lack of mitotic defects, the steady state concentration of cyclin A and cyclin B proteins in our *Rpt1^{P1}* mutant pupae was comparable with the concentration of cyclins A and B in wild type pupae.

The hypomorphic nature of the *Rpt1^{P1}* mutant suggested us to seek for genetic interactions between this mutation and distinct proteasome and APC/C subunit mutations. We were able to find synergistic genetic interactions between both the *Rpt1^{P1}-ΔRpn10/S5a/p54* and the *Rpt1^{P1}-mks¹* mutations. The *mks¹* is a hypomorphic mutation of the Cdc27 subunit of the APC/C and is pharate adult lethal. The *ΔRpn10/S5a/p54* deletion, which eliminates the major multiubiquitin receptor of the proteasome, is pupal lethal. The animals which were homozygous for both mutations were L3 lethals in case of *ΔRpn10/S5a/p54*, and white pupal lethal in case of *mks¹*.

Conclusions

As a conclusion we can lay down that:

1. We characterized five distinct alleles of the Rpt1 gene.
2. We discovered a presumably lethal amino acid exchange in the C terminal region of the Rpt1 protein.
3. We created a new, hypomorphic Rpt1 mutation.
4. With the help of the hypomorphic mutation we discovered:
 - the multiphosphorylation of the Rpt1 protein
 - the role of Rpt1 in the assembly of the 26S proteasome
 - the association of Rpt1 onto the chromatin
 - the role of Rpt1 in the expressional regulation of other proteasomal subunits
 - the contribution of Rpt1 to the degradation of the Rad23B protein
 - the lack of mitotic blockage and the accumulation of multiubiquitylated proteins in the hypomorphic mutant
 - genetic interactions between subunits of the regulatory complex and a subunit of the APC/C.

Full papers

1. Kiss P, **Szabó Á**, Hunyadi-Gulyás É, Medzihradszky KF, Lipinszki Z, Pál M, Udvardy A (2005) *Zn²⁺-induced reversible dissociation of subunit Rpn10/p54 of the Drosophila 26 S proteasome*. Biochemical Journal; 391(Pt 2):301-10. IF: 4,224
2. **Szabó Á**, Pál M, Deák P, Kiss P, Újfaludi Zs, Pankotai T, Lipinszki Z and Udvardy A (2007) *Molecular characterization of the Rpt1/p48B ATPase subunit of the Drosophila melanogaster 26S proteasome*. Molecular Genetics and Genomics (under publication) IF:2,632