PhD THESES

THE POTENTIAL EXTRAPROTEASOMAL ROLE OF p54, THE UBIQUITIN RECEPTOR OF THE *DROSOPHILA* 26S PROTEASOME

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Introduction

The ubiquitin-proteasome system is responsible for the controlled proteolysis of intracellular proteins. The first component of this system, the ubiquitinating enzyme cascade recognizes the different degradation signals present in short-lived proteins and, by attaching a multiubiquitin chain, marks these proteins for degradation. Multiubiquitinated proteins are recognized and selectively degraded by the 26S proteasome, which is the second main component of the ubiquitin-proteasome system. In an ATP-dependent reaction, this large multiprotein complex is assembled from two subcomplexes: the catalytic core particle (CP) and the regulatory particles (RPs). The CP is a non-specific protease, which cannot discriminate between multiubiquitinated and non-ubiquitinated proteins.

The RPs, which can be split into base and lid subcomplexes, are attached to one or both ends of the CP, ensure the selectivity of the 26S proteasome for multiubiquitinated proteins, unfold the substrate proteins by their chaperone-like activity, open the gated channel of the CP, reprocess the ubiquitin residues of the substrate proteins and feed them into the CP. Six ATPase subunits of the RP, forming a heterohexameric ring, mediate most of these ATP-dependent reactions. The ATPase ring, together with three non-ATPase subunits, forms the base subcomplex, while the lid subcomplex is composed entirely of non-ATPase subunits. One of the lid subunits, Rpn11, in *Drosophila* p37B, which contains a novel Zn^{2+}-metalloprotease domain, is responsible for reprocessing the ubiquitin moieties of the multiubiquitinated substrate proteins. The deubiquitinating activity of Rpn11, which is strictly coupled to substrate degradation, is dependent on the unimpaired Zn^{2+}-isopeptidase function of the subunit. Removal of Zn^{2+} or mutation of the active site histidines suspends the deubiquitinating activity and stabilizes the substrate proteins.

Another important subunit of the base subcomplex is the p54 protein, which is the ubiquitin receptor of the proteasome. The selective recognition and binding of multiubiquitinated proteins are the primary and, from the aspect of cellular homeostasis, the most critical functions of the RPs. The p54 subunit of the RP fulfils all the criteria of an ubiquitin receptor. Although the cooperation of p54 and other proteasome-interacting proteins in multiubiquitinated substrate recognition has been extensively analyzed, one feature of the mode of action of p54 in the substrate selection still awaits clarification. There are two alternative scenarios for the mode of

substrate selection and binding. If it is assumed that p54 is located on the surface of the regulatory particle in an exposed configuration, substrate selection may proceed in the firmly bound state of this ubiquitin receptor subunit. However, since p54 is the only RP subunit which exists in RP-bound and free forms in most organisms, a shuttling cycle of this subunit may be presumed during substrate selection: following its dissociation, the free subunit recruits multiubiquitinated substrates and, by reassociation with the RP, targets them for destruction.

Specific aims

The idea to examine the effect of Zn^{2+} on the structure and function of the *Drosophila* 26S proteasome came from the discovery that removal of Zn^{2+} from the Zn^{2+}-isopeptidase domain of subunit Rpn11 not only destroys the deubiquitinating activity of this subunit but also suspends the whole proteasomal degradation cycle. This finding suggested to us that certain function(s) of the 26S proteasome might be orchestrated through Zn^{2+}-coordinated interactions of RP subunits.

To address this question, we aimed to carry out complex structural and functional analysis to determine:

- whether there are Zn^{2+}-dependent interactions between the RP subunits
- whether these interactions have an affect on the proteasome function.

For this purpose, we intended to examine:

- the effect of exogenous Zn^{2+} and the effect of the removal of the exogenous Zn^{2+} on the structure and function of the *Drosophila* 26S proteasome *in vitro*,
- to model the dissociation-association process *in vivo*, by establishing and analyzing transgenic *Drosophila melanogaster* stocks expressing the full-length p54 or its derivatives.

Our final goal is to get closer to the understanding of the role of p54 in the shuttling cycle of multiubiquitinated substrate selection and to explore the extraproteasomal fate of this subunit.

Methods

Biochemical methods:

Recombinant DNA technology:

- for cloning of the Hsp82 heat-shock protein and for preparing the affinity tagged constructs
- for construction of vectors used for P-element transformation of the *Drosophila* stocks

Native polyacrylamide gel electrophoresis

- for the structural analysis of the proteasome

Denaturing polyacrylamide gel electrophoresis, Western blot analysis, Silver staining

- for identification of proteins

Two-dimensional gel electrophoresis

- for separation of the modified forms of p54

Chemical cross-linking

- for analysis of the interactions between proteasome subunits

Immunprecipitation

- for identification of the p54-interacting proteins *in vitro*

Yeast two-hybrid analysis

- for identification of the p54-interacting proteins *in vivo*

Gel filtration chromatography

- to assess whether the recombinant p54 derivatives are able to incorporate into the proteasome

Zn^{2+} affinity chromatography

- to assess whether the different proteasomal subunits contain Zn^{2+}-binding sites

Strep-Tactin affinity chromatography

- for purification of the Strep-tagged p54 derivatives

Chitin affinity chromatography

- for purification of the chitin-binding domain-p54 fusion proteins

Genetic methods:

P-element transformation

- for introduction of our transgenes into *Drosophila* embryos

Applications of the Gal4-UAS system

- for specific induction of our transgenes

Results

I. Zn^{2+} induces disassembly of the 26 S proteasome and dissociation of the ubiquitin receptor (p54) subunit

To examine the presumed Zn^{2+}- dependent subunit interactions our strategy was to test the effect of exogenous Zn^{2+} on the subunit interactions of the RP. To prove this assumption first we had to confirm, that besides Rpn11, there are other RP subunits which possess Zn^{2+}-binding sites. For this purpose, subunits of highly purified 26S proteasome were dissociated under denaturing conditions and loaded on to a Zn^{2+}-charged metal chelating fractogel column. The column bound proteasome subunits eluted at approx. 0.2 M imidazole, indicating the presence of strong Zn^{2+}-binding sites on several proteasomal subunits.

We attempted to trace the presumed Zn^{2+}-dependent interactions of RP subunits by chemical cross-linking with a bifunctional protein cross-linker and with native polyacrylamid gel electrophoresis technique.

To test the effect of exogenous Zn^{2+} on the subunit interactions of the RP, a highly purified 26S proteasome preparation was preincubated with increasing concentrations (0–200 mM) of $ZnCl_2$. After this preincubation period, the RP subunits were cross-linked with disuccinimidyl-suberate. The cross-linking patterns of the RP subunits were analyzed on immunoblots developed with different RP subunit-specific monoclonal antibodies. We found that excess Zn^{2+} induced characteristic rearrangements in the subunit interactions of the lid subcomplex. The subunit interactions within the ATPase ring were not influenced, whereas all the contacts of the ubiquitin receptor subunit p54 characteristic of the native proteasome were

suspended. These data suggested that subunit p54 dissociated from the RP in the presence of exogenous Zn^{2+}, subsequently suspending all its interactions with other RP subunits.

To test this assumption a partially-purified 26S proteasome fraction was preincubated with or without 200 μM $ZnCl_2$ and fractionated on a native polyacrylamide gel containing ATP and prepared with or without Zn^{2+}, respectively. Immunoblot analysis of the control sample revealed the conventional native polyacrylamide gel pattern: the two 26S bands, corresponding to doubly-capped and singly-capped 26S proteasomes, respectively, and the free CP. Both 26S bands exhibited strong peptidase activity in the fluorigenic gel overlay assay. A completely different pattern was obtained in the presence of Zn^{2+}. The two forms of the 26S proteasome were not observed, the total amount of the RP and the CP was present as free particles and all of the p54 subunits dissociated from the RP. Overlay assay revealed the loss of peptidase activity in the presence of Zn^{2+}.

II. Reversibility of the Zn^{2+}-induced structural and functional changes in the 26 S proteasome

Removal of Zn^{2+}, which was achieved by the addition of 200 μM 1,10-phenathroline, revealed that both electrophoretic variants of the 26S proteasome were reformed upon Zn^{2+} removal. Subunit p54 was reincorporated into the reassembled 26S proteasome, and the peptidase activity of the 26S proteasomes was restored.

III. Identification of p54-interacting proteins

The Zn^{2+}- induced dissociation of subunit p54 and the potential interactions of the free subunit with cellular proteins were examined by the cross-linking technique. The cross-linked p54-interacting proteins were purified by immunprecipitation and analyzed by mass spectrometry. The heat shock protein Hsp82 and the Smt3 SUMO-activating enzyme were identified among the cross-linked partners of the dissociated p54 protein.

We have demonstrated the interaction between the p54 protein and the Smt3 SUMO-activating enzyme also by the yeast two-hybrid technique. Using the same technique the interaction of the p54 protein and the DmUbc9 SUMO-conjugating enzyme has been

demonstrated as well. Both proteins and also the Hsp82 interacted with p54 in pull-down assays. These findings further confirmed the physiological relevance of our mass-spectrometry data.

IV. Establishment and analysis of the transgenic *Drosophila melanogaster* stocks

In order to examine the shuttling cycle of subunit p54 *in vivo* we established transgenic *Drosophila melanogaster* stocks.

The p54 protein has two important domains: the von Willebrand type A domain located at the N-terminal and the two UIMs (ubiquitin interaction motifs) located at the C-terminal part of the p54 protein. According to the data found in the literature, the N-terminal half of p54 is responsible for the incorporation of p54 into the RP, and the UIM domains are responsible for the recognition of multiubiquitinated proteins. We were interested in the effect of these domains *in vivo*: what happens when we overexpress them and the full-length protein in *Drosophila.* To achieve this goal we established transgenic *Drosophila melanogaster* stocks, which express the different halves of p54 in developmental- and/or tissue-specific manner. Flies expressing the N-terminal half of p54 in every tissue during the whole development had no phenotypic effects, although these animals expressed the N-terminal half of this protein at high levels. We have demonstrated, by means of gel filtration chromatography that the N-terminal part of p54 is able to incorporate into the proteasome. In contrast with these findings, flies expressing the C- terminal half of subunit p54 died in the L3 larval stage. These animals expressed the C-terminal half of subunit p54 from the early embryonic stage through the whole development also at high levels. Dissection of these mutant larvae revealed to have smaller brain and imaginal discs compared to that of the control stock, a phenotype that reflects on an impaired proteasome function. The most striking phenotype observed with these larvae is the dilatation of the foregut, which among others can be the effect of hindered absorption or peristalsis.

Our *in vitro* data showed that the dissociated p54 protein interacts with cellular proteins, some of which have a role in the reincorporation of p54 into the proteasome (Hsp82), whereas others may have a role in the posttranslational modification of this protein (Smt3 SUMO-activating enzyme and DmUbc9 SUMO-conjugating enzyme). Therefore our next aim was to determine the potential postsynthetic modification(s) of the overexpressed transgenes to approach the extraproteasomal roles of p54. For this purpose we established transgenic *Drosophila*

melanogaster stocks, which express affinity tagged forms of subunit p54. We chose the Strep-tag, which is a short tag enabling rapid one-step purification of the protein of interest under physiological conditions. The full-length p54, its N-terminal-or C-terminal halves were expressed in Strep-tagged forms.

Similar to the untagged forms, flies expressing the N-terminal half of p54 and also the full-length protein are viable and show no phenotypic effects, whereas some fly stocks expressing the Strep-tagged C-terminal half of p54, similar to those expressing the untagged form, died in the L3 larval stage and some others died in the pupal stage. These differences in the viability can be due to the difference in the expression levels between the transgenic stocks.

We also established fly stocks which express the Strep-tagged C-terminal half of p54 in a heat inducible manner.

V. Identification of the postsynthetically modified forms of the C-terminal half of p54

We have performed Strep-Tactin affinity purification of the induced Strep-tagged C-terminal half of subunit p54 from larvae and from non-induced control larvae. We have found that several higher molecular weight derivatives of the transgenic protein accumulate in the induced larvae. These accumulated forms, which are posttranslationally modified forms of the p54 protein, are completely absent in the non-induced control strains and are presumably responsible for the lethality. Therefore in the next experiments we concentrated on the identification of these posttranslationally modified forms of p54. The C-terminal half of p54 and its postsynthetically modified derivatives were purified on Strep-Tactin Macroprep column. The eluted fractions were separated by means of a 2-D gel electrophoresis technique by which we could exploit the extreme acidic isoelectric point of the C-terminal half of p54 and its modified forms and could obtain high resolution and a preparation sufficiently pure for the mass-spectrometry analysis.

In parallel with this effort, we reconstituted the modification process *in vitro* using bacterially expressed C-terminal p54-chitin-binding domain fusion protein. The recombinant protein was immobilized on chitin-beads and incubated in the presence of ATP with or without an extract prepared from *Drosophila* embryos.

MALDI-TOF mass spectrometry data revealed that the *in vivo* and also the *in vitro* modified forms of the p54 protein are ubiquitinated.

Conclusions

As a conclusion we claim that:

1. In the presence of high concentration of Zn^{2+} the *Drosophila* 26S proteasome disassembles into RPs and CP, subunit p54 dissociates from the proteasome and the peptidase activity of the 26S proteasome is lost.
2. We demonstrated that the Zn^{2+}-induced structural and functional changes are fully reversible.
3. We identified the interacting partners of the dissociated p54 protein.
4. In order to examine the shuttling cycle of subunit p54 we established transgenic *Drosophila melanogaster* stocks and during their analysis we have found that:
    - overexpression of the full-length p54 and its N-terminal half does not cause any detectable pheotypic changes
    - overexpression of the C-terminal half of the p54 protein causes lethality in the L3-pupal stages
    - the N-terminal half of p54 and the full-length protein are able to incorporate into the proteasome, while the C-terminal half of p54 is not
    - several higher molecular weight derivatives of the transgenic protein accumulate in the larvae expressing the C-terminal half of p54
    - these accumulated, posttranslationally modified forms of the p54 protein are its ubiquitinated forms.

<u>Full papers</u>

1. **Kiss P**, Szabó Á, Hunyadi-Gulyás É, Medzihradszky KF, Lipinszki Z, Pál M, Udvardy A (2005) *Zn^{2+}-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; 278(1):17-29. IF:2,632