Developmental-stage-specific regulation of the polyubiquitin receptors in *Drosophila melanogaster*

Zoltán Lipinszki¹, Petra Kiss¹, Margit Pál¹, Péter Deák¹, Áron Szabó¹, Eva Hunyadi-Gulyás², Eva Klement², Katalin F. Medzihradszky² and Andor Udvardy¹,*

¹Institute of Biochemistry and ²Proteomics Research Group, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, PO Box 521, Hungary

*Author for correspondence (udvardy@brc.hu)

Accepted 3 June 2009

Journal of Cell Science 122, 3083-3092 Published by The Company of Biologists 2009
doi:10.1242/jcs.049049

Summary

Recognition of polyubiquitylated substrates by the proteasome is a highly regulated process that requires polyubiquitin receptors. We show here that the concentrations of the proteasomal and extraproteasomal polyubiquitin receptors change in a developmentally regulated fashion. The stoichiometry of the proteasomal p54/Rpn10 polyubiquitin receptor subunit, relative to that of other regulatory particle (RP) 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. Depletion of the polyubiquitin receptors at the end of embryogenesis is due to the emergence of a developmentally regulated selective proteolytic activity. To follow the fate of subunit p54/Rpn10 in vivo, transgenic *Drosophila melanogaster* lines encoding the N-terminal half (NTH), the C-terminal half (CTH) or the full-length p54/Rpn10 subunit were established in the inducible Gal4-UAS system. The daughterless-Gal4-driven whole-body expression of the full-length subunit or its NTH did not produce any detectable phenotypic changes, and the transgenic products were incorporated into the 26S proteasome. The transgene-encoded CTH was not incorporated into the 26S proteasome, caused third instar larval lethality and was found to be multi-ubiquitylated. This modification, however, did not appear to be a degradation signal because the half-life of the CTH was over 48 hours. Accumulation of the CTH disturbed the developmentally regulated changes in subunit composition of the RP and the emergence of the selective proteolytic activity responsible for the depletion of the polyubiquitin receptors. Build-up of subunit p54/Rpn10 in the RP had already started in 84-hour-old larvae and reached the full complement characteristic of the non-larval developmental stages at the middle of the third instar larval stage, just before these larvae perished. Similar shifts were observed in the concentrations of the Rad23 and Dsk2 polyubiquitin receptors. The postsynthetic modification of CTH might be essential for this developmental regulation, or it might regulate an essential extraproteasomal function(s) of subunit p54/Rpn10 that is disturbed by the expression of an excess of CTH.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/17/3083/DC1

Key words: Polyubiquitin receptors, 26S proteasome, Regulatory particle, Multiubiquitylation

Introduction

The ubiquitin-proteasome system is responsible for the controlled intracellular degradation of short-lived or misfolded proteins. The selective recognition of these proteins is ensured by members of the ubiquitylation enzyme cascade. Ubiquitin-conjugating enzymes and ubiquitin ligases, alone or in combination, are responsible for substrate recognition. Through their catalytic activities, the selected proteins are marked for intracellular degradation by the covalent attachment of a polyubiquitin chain. Polyubiquitylated proteins are selectively recognized and degraded by the 26S proteasome, a large proteolytic complex that is assembled in an ATP-dependent reaction from two distinct subcomplexes: the regulatory particle (RP) and the catalytic particle (CP) (reviewed by DeMartino and Gillette, 2007). The CP is a nonspecific protease – it cannot discriminate between polyubiquitylated and non-ubiquitylated proteins. The strict specificity of the 26S proteasome for polyubiquitylated proteins, which is absolutely essential for the maintenance of cellular integrity, is ensured by the RP. Additionally, RPs are responsible for unfolding the substrate proteins via their anti-chaperone activity, opening the gated channel of the CP, reprocessing the ubiquitin residues of the substrate proteins, and feeding the substrate into the CP. The contributions of the individual RP subunits to the different steps of the proteolytic process are far from clear.

The mechanism that ensures the selective recognition of the polyubiquitin chain by the RPs is a crucial step in controlled intracellular protein degradation, and has been studied in detail. Two different classes of proteins have been identified that fulfil all the criteria required for a polyubiquitin receptor. One of them, the Rpn10/S5a/p54 protein (yeast/human/Drosophila orthologs), can bind both polyubiquitylated substrate proteins and the polyubiquitin chain with high affinity in different in vitro systems (Deveraux et al., 1994; van Nocker et al., 1996; Haracska and Udvardy, 1997; Fu et al., 1998). Because it is a stoichiometric subunit of the RP, Rpn10/S5a/p54 was considered to be an obvious candidate as the polyubiquitin receptor of the RP. Its role as a polyubiquitin receptor, however, has been questioned in consequence of the observation that deletion of the yeast *Rpn10* is not lethal, produces only mild phenotypic changes and does not cause the accumulation of polyubiquitylated proteins in yeast cells (van Nocker et al., 1996).
The discovery of a group of proteins (Rad23, Dsk2 and Ddi1) containing ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains seemed to resolve this contradiction (reviewed by Hartmann-Petersen and Gordon, 2004). The UBA of these proteins ensures the selective recognition and binding of the polyubiquitin chain, whereas the UBL, by its ability to bind to the RP, performs the targeting of the polyubiquitylated proteins to the 26S proteasome. Accumulation of polyubiquitylated proteins in rpn10Δ and dsk2Δ yeast mutants promoted the view that these non-proteasomal proteins might function as polyubiquitin receptors (Wilkinson et al., 2001). Following more detailed studies, however, serious contradictions emerged concerning the exclusive role of UBA-UBL proteins as polyubiquitin receptors. In rad23ΔΔp54Δ double-mutant yeasts, more pronounced pleiotropic defects were observed than in either single mutant, and the canavanine-sensitivity of the double-mutant strain was about 100-fold higher than that of either single mutant (Lamberton et al., 1999; Elsasser et al., 2004; Verma et al., 2004). This observation suggested that the polyubiquitin receptor function requires some kind of cooperation between the Rpn10 RP subunit and a UBA-UBL protein. In vitro reconstitution of the 26S proteasome-dependent degradation of the polyubiquitylated Sic1 (UbSic1) protein confirmed this hypothesis (Verma et al., 2004). 26S proteasomes prepared from rad23Δ or rpn10Δ yeast mutants failed to bind and degrade UbSic1. Recombinant Rpn10 or Rad23 complemented the degradation failure of the respective mutant proteasomes, but the complementation of rad23Δ required the presence of at least the N-terminal von Willebrand factor A (VWA) domain of Rpn10 in the RP, in addition to Rad23. This observation clearly supported the assumption that the interaction of Rpn10 with a UBA-UBL protein is essential for the targeting and degradation of the polyubiquitylated proteins. The physiological significance of these in vitro studies was further confirmed by in vivo analysis of the degradation of relevant proteasomal substrate proteins. These in vivo studies demonstrated that an unexpected degree of specificity of the polyubiquitin receptors was necessary for the in vivo degradation of different polyubiquitylated proteins (Verma et al., 2004).

Analysis and comparison of the in vivo and in vitro data, however, hint that alternative, hitherto-unresolved mechanisms might complicate the events of the substrate recognition cycle. Thus, the mechanism proposed for the recognition and targeting of polyubiquitylated proteins does not explain the phenomenon that the degradation of polyubiquitylated proteins is severely inhibited in the presence of an excess of S5a (Deveraux et al., 1995) or Rpn10 polyubiquitin receptors (Verma et al., 2004). Furthermore, the reversible dissociation-association observed in vitro for the yeast (Babbitt et al., 2005) or Drosophila (Kiss et al., 2005) proteasomal polyubiquitin receptor subunits (Rpn10 or p54, respectively), which accompanies the in vitro assembly-disassembly process of the 26S proteasome, indicates either that the recognition and targeting of polyubiquitylated proteins is a more complex process, or that the proteasomal polyubiquitin receptors have a yet-unknown extraproteasomal function(s). The latter is supported by the observation that, in both Drosophila (Haracska and Udvardy, 1995) and yeast (van Nocker et al., 1996), some of the proteasomal polyubiquitin receptors are found in a proteasome-unassociated pool. The presumed extraproteasomal role of Rpn10 and its extraproteasomal interaction with the Dsk2 polyubiquitin receptor are supported by the in vivo observation that the severe cytotoxicity of Dsk2 overexpression in yeast is mitigated by the accumulation of extraproteasomal Rpn10 (Matiuhin et al., 2008). The CTH of Rpn10, carrying the ubiquitin-interacting motif (UIM) is alone responsible for this compensatory function, suggesting that in its extraproteasomal state the UIM of Rpn10 might mask the UBL domain of Dsk2, competing in this way with the interaction of Dsk2 with the proteasome. However, the presumed extraproteasomal shuttling of the proteasomal polyubiquitin receptor is questioned by the observation that human 26S proteasomes degraded polyubiquitylated Sic1 and c-IAP1 proteins in vitro without dissociating into the RP and CP and releasing any subunit (Kriegenburg et al., 2008).

In the present work, the presumed shuttling cycle of the Drosophila proteasomal polyubiquitin receptor subunit (p54) was studied by exploiting the observation that the stoichiometry of the RP subunits exhibits characteristic developmental changes. As compared with embryos, there is a sudden drop in the relative concentration of subunit p54 in the RP during the first instar larval stage; the concentration remains low throughout the larval stages, starts to increase at the end of the third instar larval stage, and again reaches a high level in pupae and adults. To investigate the regulatory events leading to the down-regulation of subunit p54 during the larval stages, transgenic Drosophila stocks were generated to express different domains of subunit p54, and the intracellular fates of both the transgenic proteins and the endogenous p54 subunit were followed.

Results

Developmental fate of subunit p54

For an analysis of the fate of subunit p54 in synchronously developing wild-type D. melanogaster, eggs were laid on agar plates for 1 hour at 25°C and incubated at this temperature for various times. Total protein extracts were prepared from animals in different developmental stages, and the subunit compositions of the 26S proteasomes were analyzed by an immunoblotting technique, using a mixture of monoclonal antibodies recognizing the p48A (Rpt3) ATPase subunit of the RP base, the p42A (Rpn7) and p39A (Rpn9) subunits of the RP lid, three different monoclonal antibodies recognizing three different epitopes (see below) located in the CTH of subunit p54 (Rpn10), and a polyclonal antibody developed against the CP. The loading of equal amounts of total proteins from the different developmental stages (Fig. 1A) revealed that the stoichiometry of subunit p54 dropped dramatically during the first instar larval stage, remained low throughout the larval stages, started to increase at the end of the third instar larval stage, and reached the same high level in pupae and adults as is characteristic for the early embryos (Fig. 1B).

Individually, all three anti-p54 monoclonal antibodies, recognizing three different epitopes of the subunit, detected the same developmental switch (data not shown). Thus, it is very unlikely that a change of a postsynthetic modification recognized by a monoclonal antibody is responsible for the developmental switch. There was no detectable fluctuation in the stoichiometry of other RP or CP subunits during the development of Drosophila. Although a fraction of the p54 subunit is present in a proteasome-unassociated pool in the ATP-depleted embryonic extract (Haracska and Udvardy, 1995), the sudden drop in the p54 content of the RP in the larvae is not due to the removal of this proteasome-unassociated p54 pool during the embryo-larva developmental transition, because only trace amounts of p54 protein were present extraproteasomally in total embryonic protein extracts prepared in the presence of ATP and fractionated on a Superose 6 sizing column in ATP-containing buffer (supplementary material Fig. S1).
Reduction of the concentration of subunit p54 during the larval developmental stages might, in principle, be compensated by overexpression of the non-proteasomal polyubiquitin receptors. This is not the case, however, at least for the two major non-proteasomal polyubiquitin receptors. As shown in Fig. 1C, there was a serious reduction in the concentrations of the Rad23 and Dsk2 proteins during the larval stages, and the developmental profile of this down-regulation was very similar to that observed for p54. This suggests that the polyubiquitin receptors of the ubiquitin-proteasome system are down-regulated during larval development. In contrast to the serious reduction in the concentrations of the proteasomal and extraproteasomal polyubiquitin receptors, there was no detectable accumulation of the polyubiquitylated proteins during the early larval stages (supplementary material Fig. S2), probably because of the very low mitotic activity in the larvae. Our unpublished results have revealed that two other yeast extraproteasomal polyubiquitin receptor orthologs, the UBA-UBL protein Ddi1 [CG4420] and the Pru domain containing Rpn13 [CG13349, p42E], have only weak affinity for polyubiquitylated proteins in D. melanogaster (Z.L., M.P., P.D. and A.U., unpublished results). For this reason, only the two major known polyubiquitin receptors were examined in this work.

To test whether transcriptional regulation of the genes encoding the individual RP subunits is responsible for the depletion of subunit p54 in the larvae, total RNA was prepared from all the developmental stages analyzed above, and the changes in the relative concentrations of the mRNAs encoding the p54/p48B ratio in the first instar larvae as compared to embryos, and this ratio seemed to remain constant during the later larval stages. The p54/p48B mRNA ratio increased slightly in the pupae and adults (Fig. 1D). This observation clearly indicates that the depletion of subunit p54 in first instar larvae is regulated either at the level of p54 mRNA translation, or by a selective proteolytic degradation of the p54 protein. Our results support this latter assumption.

The selectivity of the postulated proteolytic degradation should ensure the elimination of a limited set of proteins, exclusively during the early larval developmental stages. To test this assumption, an aliquot of total embryonic protein extract was incubated at 25°C for 10, 30 or 60 minutes with total protein extracts prepared from synchronized 60-, 72-, 84-, 96-, 108- or 120-hour-old larvae. Embryonic protein extract incubated at 25°C for 60 minutes without

---

Fig. 1. Developmental changes in the subunit composition of the RP. (A) Equal amounts of total proteins prepared from different developmental stages of wild-type D. melanogaster were fractionated on 9% SDS-polyacrylamide gel and analyzed by silver staining. (B) The same amount of proteins were analyzed by immunoblotting with a mixture of monoclonal antibodies recognizing p54, p48A, p42A and p39A RP subunits, and with a polyclonal antibody developed against the CP. (C) Lanes 1-15. Immunoblot analysis with same amount of protein samples described above using a mixture of polyclonal anti-Rad23 and anti-Dsk2 antibodies. Lane 16: total embryonic protein extract reacted only with anti-Rad23 antibody. Lane 17: total embryonic protein extract reacted only with anti-Dsk2 antibody. (D) Semi-quantitative RT-PCR analysis of p54 and p48B mRNAs in different developmental stages of D. melanogaster. rpL17A ribosomal protein mRNA served as loading control.
any larval extract served as control. The reactions were terminated by boiling the samples in SDS sample buffer, and the changes in the subunit pattern of the 26S proteasomes were analyzed using an immunoblotting technique. In these reaction mixtures, the embryonic 26S proteasome molecules provided most of the p54 subunit. As shown in Fig. 2A, protein extracts derived from 60-, 72-, 84- and 96-hour-old larvae selectively degraded the p54 subunit, leaving all the other proteasomal subunits intact. The selectivity of this proteolytic degradation was observed by the separation of glycogen phosphorylase and β-importin proteins (present in both the embryonic and the larval protein extracts) remained completely intact during this incubation, and no detectable changes were noted in the silver-stained total protein profile of the incubated samples (Fig. 2B). This is in sharp contrast to the selective degradation of Dsk2 and Rad23 polyubiquitin receptors in early larval protein extracts (Fig. 2C). The proteasome inhibitor MG132 reduced, whereas the truncated subunit was undetectable in embryos, pupae or adults. The truncated N-terminal fragment co-eluted with the larval 26S proteasomes following size fractionation on Superose 6, indicating that the fragment is an integral component of the proteasome (data not shown).

Analysis of the in vitro degradation of subunit p54 by second instar larval protein extracts revealed another level of selectivity. In the immunoblotting experiments illustrated in Fig. 1B and Fig. 2A, the p54 subunit was detected by three monoclonal antibodies that recognize the CTH of the subunit. To analyze the fate of the NTH that carries the VWA domain required for the incorporation of the subunit into the RP, the samples analyzed in Fig. 2A were re-probed with a polyclonal antibody developed against recombinant NTH of p54. A typical experiment with the 60-hour-old larval extract demonstrates that, in vitro, the N-terminal fragment of the proteasome-bound subunit p54 is highly protected against proteolytic attack (Fig. 2D). The size of the protected polypeptide is about 28 kDa, i.e. the whole VWA domain is protected. To follow the physiological relevance of this observation, the protein samples of synchronously developing wild-type D. melanogaster (presented in Fig. 1B) were re-probed with the NTH-specific polyclonal antibody (Fig. 2E). The protected polypeptide fragment detected in the in vitro degradation assay was present in all the larval developmental stages in which the full-length p54 was substantially reduced, whereas the truncated subunit was undetectable in embryos, pupae or adults. The truncated N-terminal fragment co-eluted with the larval 26S proteasomes following size fractionation on Superose 6, indicating that the fragment is an integral component of the proteasome (data not shown).

**Ectopic expression of different domains of subunit p54**

For our study of the regulatory events leading to the down-regulation of subunit p54 in the larvae, transgenic Drosophila stocks were generated to express different domains of subunit p54, and the fate, the postsynthetic modification and the phenotypic effect of the ectopic expression of the transgenes were studied.

Transgenic constructs encoding the untagged or Strep-tagged versions of the CTH, the NTH and the full-length p54 were generated in the pUAST vector. The NTH (1-613 bp of the cDNA) carries the VWA domain required for the incorporation of the subunit into the RP, whereas the CTH (614-1188 bp of the cDNA) carries the UIM motifs responsible for the recognition and binding of the polyubiquitin chain (Fu et al., 1998). Following transformation, homozygous viable and fertile transgenic stocks without any detectable abnormalities were selected. Transgene expression was induced by crossing the transgenic flies to the Gal4 driver stock. This driver ensures a high-level expression of UAS-
regulated transgenes in all cells of the transgenic animals during all developmental stages.

Four different transgenic lines carrying the Strep-tagged full-length p54 gene were crossed to daughterless-Gal4 (da-Gal4) and the viability, fertility and appearance of visible morphological abnormalities in the offspring analyzed. Expression of the Strep-tagged full-length p54 did not cause any detectable changes in the parameters tested. The eight-amino-acid-long Strep-tag allowed the discrimination and simultaneous immunodetection of the endogenous and transgenic p54 proteins. As shown in Fig. 3B,C, the Strep-tagged full-length p54 protein was expressed when induced by the da-Gal4 driver. The whole amount of the transgenic product was incorporated into the 26S proteasome: after size-fractionation of a total protein extract of da-Gal4/Strep-p54(7) stock, all the Strep-tagged transgenic protein co-migrated with the endogenous p54, the p42C (Rpt6) RP base and the p39 (Rpn9) RP lid subunits, and no free transgenic protein was detected in the monomeric protein fractions. Similar results were obtained with the other three transgenic stocks expressing the Strep-tagged full-length p54 (data not shown). Transgenic stocks expressing the NTH were also viable and fertile, which is in contrast to the fact that the truncated subunit was incorporated into the 26S proteasome (Fig. 3D). These findings are in sharp contrast to the phenotypic changes observed following the expression of the CTH.

Four different transgenic lines carrying the 3′-half of the p54 gene were tested. Crossing each of these stocks to the da-Gal4 driver resulted in third instar larval lethality. Transgene expression was analyzed by an immunoblotting technique. Transgene-encoded CTH protein was not detected in the transgenic animals before they were crossed to the Gal4 driver, but induction with the driver resulted in the accumulation of large amounts of CTH protein (Fig. 4). As expected, the transgenic CTH protein lacking the VWA domain was not incorporated into the 26S proteasome and appeared as a monomeric protein after Superose 6 fractionation (Fig. 3E). Transgenic line da-Gal4/C18, expressing the CTH at the highest level, was chosen for further studies.

It was previously shown that an excess of S5a or Rpn10 inhibited the proteasomal degradation of two different polyubiquitylated proteins in vitro (Deveraux et al., 1995; Verma et al., 2004). Thus, the lethal phenotype of CTH overexpression might be due to a severe inhibition of the in vivo turnover of polyubiquitylated proteins. To test this assumption, we analyzed the accumulation of polyubiquitylated proteins in larvae following the induction of CTH expression. As shown in Fig. 5, induction of the CTH resulted in the accumulation of polyubiquitylated proteins in da-Gal4/C18 larvae.

Postsynthetic modification of the CTH

A previous study of the Zn2+-induced reversible dissociation of subunit p54 demonstrated that, during its extraproteasomal state, this subunit interacts with several non-proteasomal proteins, including the Smt3 SUMO-activating enzyme (Kiss et al., 2005). The in vivo relevance of this interaction was confirmed by yeast two-hybrid analysis: the NTH of p54 displayed a strong interaction with the Smt3 SUMO-activating enzyme and the Ubc9 SUMO-conjugating enzyme. This observation raised the possibility that, during its presumed extraproteasomal state, this subunit becomes post-synthetically modified, which might be important for an earlier unrecognized extraproteasomal function of the subunit. As the CTH cannot incorporate into the RP, its expression might model an extended extraproteasomal state of the CTH of subunit p54, allowing the formation and, hence, the accumulation and detection of a presumed postsynthetic modification(s).

To study the occurrence of postsynthetic modification, a transgenic Drosophila stock (8SC) carrying the Strep-tagged CTH

![Diagram](https://via.placeholder.com/150?text=Diagram+of+Subunit+p54+Expression)
transgene was crossed to the da-Gal4 driver. The total protein extract was prepared from da-Gal4/8SC third instar larvae and the Strep-CTH protein was purified on a Strep-Tactin affinity column. The affinity-purified proteins were fractionated on 10% SDS-polyacrylamide gel and immunoblotted with anti-p54 monoclonal antibodies. Total protein extract prepared from non-induced 8SC larvae (i.e. before crossing to the da-Gal4 driver) served as control.

As shown in Fig. 6, a set of anti-p54 reactive proteins appeared in the affinity-purified fraction derived from the larvae expressing the Strep-tagged CTH. These proteins were completely missing in the eluted fractions purified from non-induced larvae. The most prominent of these bands, which was about 8 kDa larger than the Strep-tagged CTH, was detectable even in the total larval protein extract (see also Fig. 4). The band marked by an asterisk (Fig. 6) corresponds to a nonspecifically bound full-length p54 (control experiments with recombinant p54 proved that Strep-Tactin column can nonspecifically bind p54, data not shown). The immunoreactive bands formed a ladder, reminiscent of an ubiquitylation ladder.

To prove the presumed ubiquitylation, the affinity-purified protein fraction was further purified by two-dimensional IEF-SDS PAGE, exploiting the very acidic nature of the CTH (calculated pI is 3.6). Two parallel two-dimensional gels were prepared: one of the spots (supplementary material Fig. S3), providing direct evidence for the in vivo ubiquitylation of the CTH. Our efforts to identify the lysine residue(s) ubiquitylated in the CTH failed, due to the low quantity of purified material recovered. Ubiquitylation of the CTH can likewise be achieved in vitro by incubating the recombinant CTH in an embryonic protein extract in the presence of ATP and ubiquitin. The modified proteins were affinity-purified and analyzed by immunoblotting technique (supplementary material Fig. S4).

Ubiquitylation of the CTH is not a degradation signal
In vivo ubiquitylation of the yeast Rpn10 subunit has recently been demonstrated (Crosas et al., 2006), though the site of the ubiquitylation was not identified. The short half-life of the ubiquitylated Rpn10 (45 minutes) suggested that a bona fide polyubiquitin chain, a true degradation signal, is formed on the Rpn10 subunit. With the aid of the heat-inducible driver, we determined the half-life of the CTH protein. In this experiment, R80/da-Gal4/C18 flies grown at 19°C were heat-shocked at 30°C for 36 hours, and the incubation was then continued at 19°C for a further 4 days. Total protein extracts were prepared from non-heat-shocked and heat-shocked animals, and from animals kept for 1-4 days at 19°C following the heat-shock. Immunoblot analysis (Fig. 8) revealed that at 19°C the CTH is not expressed at all. Following 36 hours of heat shock, a large amount of the CTH had accumulated. It proved to be a stable protein not expressed at all. Following 36 hours of heat shock, a large amount of the CTH had accumulated. It proved to be a stable protein as its half-life at 19°C was over 48 hours. Thus, the in vivo ubiquitylation of the CTH does not serve as a degradation signal, but is more probably connected with an essential extraproteosomal function of subunit p54.
Ectopic expression of the CTH disturbs the developmental regulation of polyubiquitin receptors

To investigate the consequences of high-level CTH accumulation on the developmental regulation of the p54 subunit in larvae, total protein extracts were prepared from synchronously developing C18 and da-Gal4/C18 larvae, and the subunit composition of the 26S proteasome was analyzed by immunoblotting with a mixture of monoclonal antibodies as described above. In C18 larvae, as in wild-type animals, the p54 subunit was present at low molar concentration relative to other RP subunits during the early larval stages, and had started to accumulate in 108-hour-old larvae during the second half of the third instar larval stage (Fig. 9).

There are significant changes in the developmental regulation of p54 subunit accumulation in da-Gal4/C18 larvae. The accumulation of full-length p54 subunit has already started in 84-hour-old larvae, and reaches the maximal level in 108-hour-old larvae, just before these larvae start to perish. Similar results were observed with the extraproteasomal polyubiquitin receptors (Fig. 9). In the C18 stock, the concentrations of Rad23 and Dsk2 were low in the early larval stages, and started to accumulate in the middle of the third instar larval stage. In da-Gal4/C18 larvae, the accumulation of the extraproteasomal polyubiquitin receptors started during the second larval stage and reached its maximal level in the early third instar larvae.

The data in Fig. 2 indicate that depletion of the polyubiquitin receptors during the early larval developmental stages is a consequence of the emergence of selective proteolytic activity. It was reasonable to suppose that the disturbance of physiological regulation observed following the ectopic expression of CTH (Fig. 9) might be a consequence of an alteration in the developmentally regulated expression of this key proteolytic activity. To test this assumption, a total embryonic protein extract was incubated at 25°C for 15, 45 or 90 minutes with total protein extracts prepared from synchronized 60-, 72-, 84-, 96-, 108- or 120-hour-old C18 or da-Gal4/C18 larvae. Embryonic protein extract incubated at 25°C for 90 minutes without any added larval extract served as control. The reactions were terminated by boiling the samples in SDS sample buffer, and the changes in the subunit pattern of the 26S proteasomes were analyzed by an immunoblotting technique, as described above.

As shown in Fig. 10, the developmental profile of the selective proteolytic activity of the synchronized C18 larval extracts was indistinguishable from that of the wild-type larval extract. To detect even weaker proteolytic activities, the in vitro incubation of embryonic extract with larval extracts was extended for 15, 45 or 90 minutes in these experiments, and thus the p54 subunit of the embryonic 26S proteasomes was completely degraded even during the shortest incubation period. The ectopic expression of CTH in the da-Gal/C18 larvae inhibited this selective proteolytic activity in a developmentally regulated fashion. In early larval stages (60-hr-
72 hours), the proteolytic activity was very strong, completely degrading both the proteasome-bound full-length p54 polypeptide and the ectopically expressed CTH. This proteolytic activity was already significantly inhibited in 84-hour-old larvae and the inhibition was complete in 96-hour-old larvae. The degradation of Dsk2 and Rad23 was also inhibited in a similar developmentally regulated fashion (data not shown).

These in vitro degradation data are congruent with the in vivo changes of the proteasomal subunit patterns induced by the ectopic expression of CTH (Fig. 9). All of the data presented indicate that the accumulation of the CTH protein and/or its ubiquitylation influence the normal developmental fate of the polyubiquitin receptors in D. melanogaster by altering the developmentally regulated expression of a selective proteolytic system.

Discussion

In D. melanogaster, there is a developmentally regulated reduction in the concentration of the proteasomal and extraproteasomal polyubiquitin receptors during the larval stages. The physiological relevance of this regulation is far from clear, but the very fast cell division activity in the embryos, the highly active controlled proteolysis required for the histolysis of larval structures during the pupal stage, and the reproductive activity of the adults might explain the need for a much higher polyubiquitin receptor concentration. Our results demonstrate that a developmentally regulated selective proteolytic activity is responsible for the changes in the concentrations of the polyubiquitin receptors. This proteolytic activity emerges in the early larvae and persists at a high level until the middle of the third instar larval stage, gradually disappearing thereafter.

The selectivity of this proteolytic activity displays several unusual properties. It ensures the degradation of the proteasomal and extraproteasomal polyubiquitin receptors, leaving the other proteasomal subunits, the glycogen phosphorylase and the β-importin proteins intact. Furthermore, this proteolytic activity degrades only the CTH of subunit p54 carrying the UIM motifs, but spares the NTH of the subunit that interacts with the RP of the proteasomes (Fu et al., 1998). The specificity is further supported by the observation that, both in vivo and in vitro, this proteolytic activity spares a well-defined fragment of subunit p54 of equal length.

Transgenic expression of the full-length p54 or its NTH does not cause any detectable phenotypic changes in Drosophila, because the cellular concentrations of these transgenic proteins are strictly controlled. This follows from the observation that these transgenic proteins do not accumulate in the monomeric, extraproteasomal pool, the molar concentration of the endogenous and transgenic proteins together corresponding to the level required for the stoichiometric assembly of the RP (Fig. 3). The presence of the VWA domain in the full-length transgenic p54 protein and in its NTH ensures their incorporation into the RP. The excess transgenic products are probably degraded, and the lack of unassembled transgenic proteins might explain the viability and fertility of these transgenic animals. The CTH cannot assemble into the RP due to the lack of the VWA domain and, as it has a fairly long half-life, the CTH accumulates in large quantity in the extraproteasomal pool.

All the in vitro and in vivo data so far published demonstrate that any shift in the balance of the concentrations of the proteasomal and extraproteasomal polyubiquitin receptors results in a serious disturbance of the ubiquitin-proteasome system, which is always manifested in the accumulation of polyubiquitylated proteins (Deveraux et al., 1995; Ortolan et al., 2000; Kleijnen et al., 2000; Raasi and Pickart, 2003; Verma et al., 2004; Matiuhin et al., 2008). Although our data confirm these observations, we have noticed a hitherto-unexpected consequence of the accumulation of the CTH protein: the deregulation of the developmental program responsible for adjustment of the concentrations of proteasomal and extraproteasomal polyubiquitin receptors. RT-PCR analysis revealed that the drop in the p54 content of the RP in the first instar larvae is not regulated at the transcriptional level. It appears more probable that, as in yeast, selective proteolytic degradation of p54 is responsible for the developmental changes.

In yeast, subunit Rpn10 is a short-lived protein; it is polyubiquitylated and degraded with a half-life of 45 minutes. The half-life of Rpn10 is regulated by the competitive action of the hul5
ubiquitin ligase, which promotes the polyubiquitylation of the subunit, and the Ub6 deubiquitylating enzyme, which removes the polyubiquitin modification (Cross et al., 2006). In Drosophila, at least during the early larval stages, the fate of subunit p54 is regulated by a different proteolytic mechanism. This follows from the observation that the in vitro degradation of subunit p54 by early larval protein extracts is resistant to the proteasome inhibitor MG132.

Our in vitro studies (Z.L., M.P., P.D. and A.U., unpublished results) reveal that the recombinant CTH can efficiently bind not only the polyubiquitylated proteins, but also the Dsk2 and Rad23 extraproteasomal receptors. Thus, the ectopic accumulation of CTH, which is a stable protein with a half-life of more than 48 hours, might sequester both the polyubiquitylated proteins and the very low amount of extraproteasomal ubiquitin receptors present in the larvae, in this way inhibiting the ubiquitin-proteasome system of the larvae completely, and leading to death of the transgenic animals.

Materials and Methods

The cDNA-encoding subunit p54, and its NTH or CTH (1-613 and 614-1188 bp), were cloned into the pASK-IBA5 vector (IBA) in frame with the Strep-tag. For construction of the Strep-tagged versions of the transgenes, the cDNA derivatives were first cloned into the pASK-IBA5 vector (IBA) in frame with the Strep-tag, and the Strep-cDNA cassettes were inserted into the pB(P[UBA]) vector. All the constructs (Fig. 3A) were verified by DNA sequencing. The plasmid constructs were injected into w1118 embryos, and transformed flies were selected according to standard procedures (Spradling, 1986). Appropriate drivers of the Gal4 system ensured the induction of transgene expression (Duffy, 2002).

To collect synchronously developing Drosophila samples, 200 virgin females from the appropriate stocks (wild-type w1118 or C18 transgenic lines) were collected and fed for 2 days on yeast-agar medium at 25°C. From the third day, they were fed for 2 days on yeast-rich agar medium at 25°C. From the third day, they were fed for 2 days on yeast-rich agar medium at 25°C. To obtain biological samples of different developmental stages, eggs were incubated at 25°C for 0-12 and 12-24 hours (embryos); 36 and 48 hours (sectored larvae); 3, 5, 7 and 9 days (early larval stages); 10 and 11 days (late larval stages); 1, 2 and 3 days (pupae); and w1118; If/CyO; P{UAST-P-strep-p54}, w1118; If/CyO; P{UAST-P-strep-p54-NTH}.

This work was supported by a grant from the Hungarian Scientific Research Fund (OTKA T046177). This publication was supported by the Dr Rollin D. Hotchkiss Foundation. The Proteomics Research Group was supported by the Hungarian National Office for Research and Technology (RET-08/2004). A participant of this consortium is Kromat Ltd. that provided the Agilent 1100 nano-LC XCT Plus Ion Trap mass spectrometer system. Special thanks to Christoph W. Turck (Max-Planck Institute of Psychiatry, Munich, Germany) who gave the opportunity to use the LTQ-Orbitrap Mass spectrometer in his laboratory.

References


Superose 6 fractionation of total embryonic protein extract (Fraction number)

12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50

- p54
- p48A
- p42A
- p39A
- CP$_\alpha$7

Superose 6 fractionation of total larval protein extract (Fraction number)

12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50

- p54
- p48A
- p42A
- p39A
- CP$_\alpha$7
<table>
<thead>
<tr>
<th>Embryo</th>
<th>Larvae</th>
<th>Pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12 hrs</td>
<td>1st instar</td>
<td></td>
</tr>
<tr>
<td>12-24 hrs</td>
<td>2nd instar</td>
<td>3rd instar</td>
</tr>
</tbody>
</table>

36 hrs  48 hrs  60 hrs  72 hrs  84 hrs  96 hrs  108 hrs  120 hrs  white  brown

anti-Ub polyclonal Ab
MALDI-TOF spectrum of the ubiquitinated p54 construct (UA2v)

Sequence coverage of the Strep-CTH construct, based on a MALDI spectrum (Matched peptides shown in **Bold Red**):

CTH

1 MASWSHPQFE KGGNVEFQGV DPNEDPELAL ALRVSMEEQQR QRQSEQRRA
51 NDPGAPPTG DASGGGAVSG SGGNREASAG AENEANTAEA MLQRALALST
101 ETPEDLNPDF ANMTQEEQIA FMQMSMQDA PDDSVTTQQAK RKPTDEANAP
151 MVDVDEYSEV IGDPFGLQSV LENLPGVPQ SEAVDDAVGS LNTKDKKKSD
201 GKSQKK

Sequence coverage of ubiquitin, based on a MALDI spectrum (Matched peptides shown in **Bold Blue**)

Ubiquitin

1 MQIFVKTTLG KTITLEVPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL
51 EDGRTLSYN IQKESSLHLV LRLRGG

Peaks marked in **green** are degradation products of chymotrypsin.
Recombinant protein extract

Affinity-purified proteins

anti-p54 mAbs

CTH-Ub$_1$
CTH-Ub$_2$
CTH-Ub$_3$
CTH-CTH
CTH

CTH