Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in *Drosophila melanogaster*

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Summary

The regulatory complex of the 26S proteasome is responsible for the selective recognition and binding of multiubiquitinated proteins. It was earlier shown that the subunit S5a/Rpn10/p54 of the regulatory complex is the only cellular protein capable of binding multiubiquitin chains in an in vitro overlay assay. The role of this subunit in substrate selection, however, is a subject of debate, following the observation that its deletion in *Saccharomyces cerevisiae* is not lethal and instead causes only a mild phenotype. To study the function of this subunit in higher eukaryotes, a mutant *Drosophila* strain was constructed by deleting the single copy gene encoding subunit S5a/Rpn10/p54. This deletion caused larval-pupal polyphasic lethality, multiple mitotic defects, the accumulation of higher multimers of ubiquitinated proteins and a huge accumulation of defective 26S proteasome particles. Deletion of the subunit S5a/Rpn10/p54 does not destabilise the regulatory complex and does not disturb the assembly of the regulatory complex and the catalytic core. The pupal lethality is a consequence of the depletion of the maternally provided 26S proteasome during the larval stages and a sudden increase in the proteasomal activity demands during the first few hours of pupal development. The huge accumulation of the fully assembled 26S proteasome in the deletion mutant and the lack of free subunits or partially assembled particles indicate that there is a highly coordinated accumulation of all the subunits of the 26S proteasome. This suggests that in higher eukaryotes, as with yeast, a feedback circuit coordinately regulates the expression of the proteasomal genes, and this adjusts the actual proteasome concentration in the cells according to the temporal and/or spatial proteolytic demands.

Key words: 26S proteasome, Regulatory complex, S5a/Rpn10/p54 subunit, Multiubiquitin binding subunit, Mitotic phenotype

Introduction

The dynamic turnover of cellular proteins is maintained by a regulated balance between protein synthesis and degradation. In the regulated and 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 (reviewed by Weissman, 2001). The same enzyme cascade is responsible for the multiubiquitination of damaged or misfolded proteins (reviewed by Davies, 2001). 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 (reviewed by Zwickl et al., 2001). 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. In the *Thermoplasma acidophilum* 20S proteasome, the orifices of this channel, which are the entry sites of substrate proteins (Wenzel and Baumeister, 1995), are situated at the bases of the barrel (Löwe et al., 1995). In the crystal structure of the *Saccharomyces cerevisiae* 20S proteasome, however, these orifices are missing, suggesting that the channel is gated in eukaryotes (Groll et al., 1997; Groll et al., 2000). In consequence of the narrowness of the central channel and the gated nature of its orifice, the catalytic centres inside the central nanocompartment of the 20S proteasome are inaccessible for folded proteins (Wenzel and Baumeister, 1995). In sharp contrast with the strict selectivity of the 26S proteasome for multiubiquitinated proteins, the 20S proteasome can efficiently degrade non-ubiquitinated proteins.

The functions of the RC can be deduced from a comparison of the enzymatic properties of the 26S proteasome with those of the catalytic core. 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 (Braun et al., 1999; Strickland et al., 2000). Unfolding of the substrate proteins is most probably an ATP-dependent step, and the six

ATPase subunits present in the RC (Dubiel et al., 1992; Dubiel et al., 1995) 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 (Köhler et al., 2001). 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 must be ensured by the RC. This assumption is supported by the observation that S5a/Rpn10/p54 [for the nomenclature of the human, yeast and *Drosophila* regulatory complex subunits, see Hölzl et al. (Hölzl et al., 2000)] is one of the RC subunits of the 26S proteasome that can recognise and bind multiubiquitin chains in vitro (Deveraux et al., 1994; Deveraux et al., 1995; Haracska and Udvardy, 1995; Haracska and Udvardy, 1997; van Nocker et al., 1996a; van Nocker et al., 1996b). More recently, in vitro crosslinking studies revealed that a reactive multiubiquitin chain can be selectively crosslinked to one of the ATPase subunits of the RC (Lam et al., 2002). The role of S5a/Rpn10/p54 in substrate recognition is debated owing to the observation that deletion of this subunit in yeast is not lethal and has only a mild phenotype (Van Nocker et al., 1996b). Deletion of this subunit in the haploid moss *Physcomitrella patens*, however, causes developmental arrest (Girod et al., 1999), and the polyubiquitin-binding site of the fission yeast homologue of S5a/Rpn10/p54 is essential when the S14/Rpn 12/p30 subunit is compromised (Wilkinson et al., 2000).

In order to gain an insight into the function of this RC subunit in higher eukaryotes, we 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.

Materials and Methods

Strains

Wild-type and mutant strains were maintained and mated on standard yeast-corn meal-agar medium and all experiments were performed at 25°C. All genetic markers used have been described previously (Lindsley and Zimm, 1992). Stocks *Gl Sb/TM3, Ser* and *yw; TM3, Sb/TM6, Hu* were kindly provided by L. Sipos and J. Gausz, respectively.

Isolation of deletions by *P*-element-induced male recombination

The *P-lacW* insertion near the 3′ end of the *pros54* gene in line 0554/18 (Deák et al., 1997) was used to generate a mutant for the *pros54* gene by the male recombination system described by Preston et al. (Preston et al., 1996). *Egfr/CyO, P(Δ2-3)* males were crossed en masse to *Gl Sb/TM3, Ser* females. From the offspring the *+/CyO, P(Δ2-3); +/Gl Sb* males were collected and crossed en masse to homozygous *yw; P-lacW*$^{0554/18}$ females. In the F0 generation, the *yw/Y; +/CyO, P(Δ2-3); Gl Sb/+ P-lacW*$^{0554/18}$ + 'jumpstarter males' were collected and crossed in groups of 4-5 to 8-10 homozygous females of *w*^{1118} genotype. In the F1 generation, the *w*^{1118}*/Y; +/+; Gl P-lacW*$^{0554/18}$ +/+ and the *w*^{1118}*/Y; +/+; + P-lacW*$^{0554/18}$ *Sb/+* recombinants were selected as single males and crossed to *yw; TM3, Sb/TM6, Hu* females. In the F2 generation, the *yw/Y; Gl P-lacW*$^{0554/18}$ *+/ TM3, Sb* males were crossed to *yw/w*^{1118}*; Gl P-lacW*$^{0554/18}$ *+/ TM3, Sb* females, or the *yw/Y; + P-lacW*$^{0554/18}$ *Sb/ TM6, Hu* males were crossed to *yw/w*^{1118}*; + P-lacW*$^{0554/18}$ *Sb/ TM6, Hu* females in order to establish stocks.

P-element-mediated transformation

The genomic *Hin*dIII, *Pst*I and *Sac*I rescue constructs (Fig. 1C) were microinjected together with the wing-clipped helper *P*-element (*wcΔ2-3*) into *w*^{1118} syncytial blastoderm stage embryos by using standard techniques, and the *P(w*$^{+}$*)* transformants of the second generation were balanced in stocks. For the rescue experiments, we used second chromosomal insertions to allow the *Df(3L)pros54P(w*$^{+}$*)* deletion on the third chromosome to become homozygous.

Lethal phase analysis

The mutant genotype lacking *pros54* [*yw; Pst I/y*$^{+}$*CyO; Df(3L)pros54P(w*$^{+}$*)/Df(3L)pros54P(w*$^{+}$*)*, see later] is a segregant of the stock *yw; Pst I/y*$^{+}$*CyO; Df(3L)pros54P(w*$^{+}$*)/TM6c,Tb Sb*. Eggs were collected from this stock for 12 hours, and the first instar larvae were collected and transferred to fresh food 24 hours later. The number of L2 and L3 larvae and puparia were determined in parallel samples after 2, 4 and 6-8 days, respectively, taking into consideration the fact that the mutant larvae developed at a lower rate. To test their developmental capacity, white puparia (both mutant and wild-type) were collected daily and transferred to a wet chamber to prevent desiccation. Ages of pupae are given in hours after white puparium formation (APF) at 25°C.

Embryo lethality was determined by counting the hatched and unhatched eggs laid by *+/yw; Pst I/+; Df(3L)pros54P(w*$^{+}$*)/+* parents derived from crossing *yw/Y; Pst I/y*$^{+}$*CyO; Df(3L)pros54P(w*$^{+}$*)/TM6c, Tb Sb* males to Oregon R females. The same experiment was repeated with *+/w*^{1118}*; Df(3L)pros54P(w*$^{+}$*)/+* and Oregon R parents.

Cytological characterisation

Brains of wandering third instar larvae were dissected in PBS and transferred into a drop of 45% acetic acid for 30 seconds. The brains were then stained in a drop of 3% aceto-orcein (dissolved in 45% acetic acid) for 3-5 minutes, and the excess stain was removed by transferring the brains into a drop of 60% acetic acid for a few seconds. Finally, the brains were transferred into a small drop of 3% aceto-orcein (dissolved in 60% acetic acid) on a coverslip, which was then picked up by touching it with a clean microscope slide. The slides were wrapped in tissue paper and squashed very hard for 10-15 seconds. The edges of the coverslip were sealed with nail polish and the preparations were observed using a phase-contrast microscope, using 40× and 100× objectives.

Protein gel electrophoresis and immunoblotting

Total protein extracts for denaturing polyacrylamide gel electrophoresis (PAGE) were prepared by disrupting embryos, larvae or pupae directly in SDS sample buffer in a microhomogeniser. The viscosity of the lysate was decreased by shearing the extract through a 27 gauge injection needle. For immunoblot analysis, proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, reacted with different subunit-specific monoclonal or polyclonal antibodies and visualised by an enhanced chemiluminescent technique, using HRP-conjugated secondary antibodies and the Supersignal-HRP chemiluminescent substrate (Pierce).

Subunit-specific monoclonal antibodies were raised in mice immunised with the purified regulatory complex. Hybridoma cell lines were selected by standard procedures (Shulman et al., 1978). The subunit specificity of the monoclonal antibodies had been characterised previously (Kurucz et al., 2002).

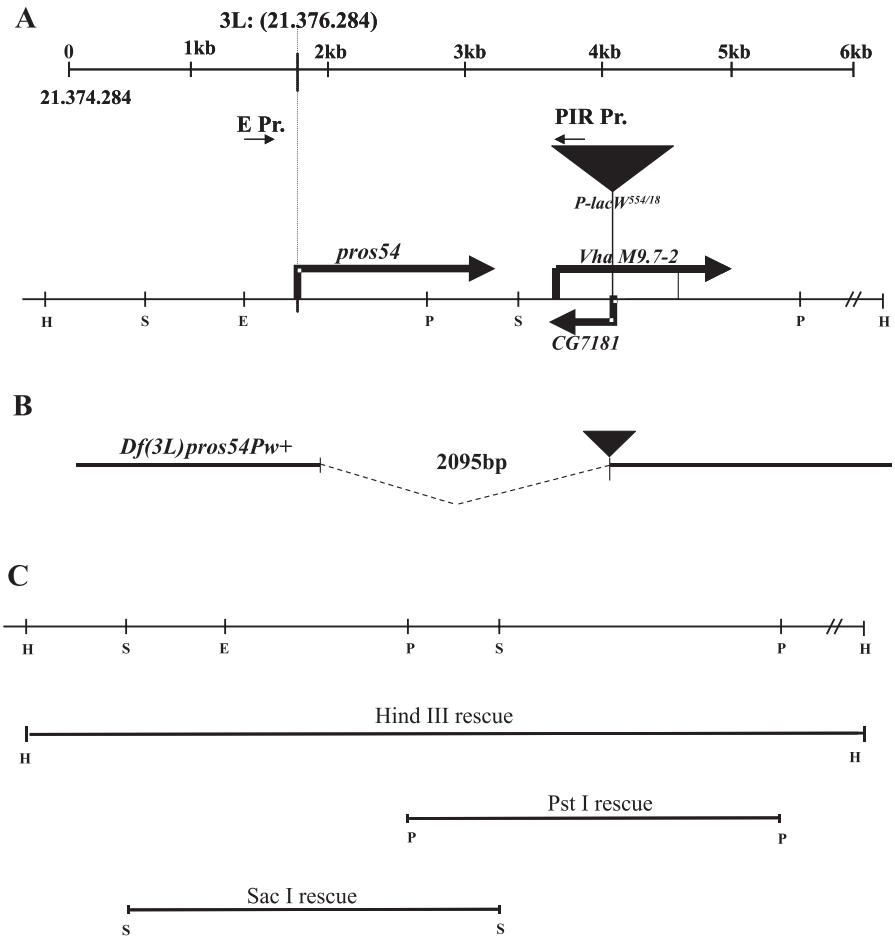

Fig. 1. Molecular map of the *pros54* genomic region. (A) Position of the *pros54*, *Vha M9.7-2* and *CG7181* genes according to GadFly. Arrows show the location and the direction of these genes. The triangle labels the site of *P-lacW*$^{0554/18}$ insertion. The E and PIR PCR primers used to screen for genomic deletions are indicated in the upper part of the figure. S, E and P are *Sac*I, *Eco*RI and *Pst*I restriction sites, respectively. Numbers in parentheses give the nucleotide-scale positions according to GadFly. (B) Extension of *Df(3L)pros54P(w*$^{+}$*)*. (C) Restriction fragments used in rescue experiments (see text).

For native polyacrylamide gel electrophoresis of total pupal protein extracts, pupae were homogenised in a solution containing 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 0.25 M sucrose. After the extract had been cleared in a microcentrifuge by centrifugation for 10 minutes, at 20,100 ***g*** at 4°C, the 26S proteasome was analysed on the single layer native polyacrylamide gel system described previously (Glickman et al., 1998a). For immunoblotting, the gels were soaked for 5 minutes at room temperature in western blotting transfer buffer supplemented with 1% SDS and transferred to a nitrocellulose membrane by a standard procedure (Sambrook et al., 1989). The in-gel dissociation of the proteasome subunits by SDS treatment greatly improved the transfer efficiency, permitting the immunodetection of the 26S proteasome from a single pupa or larva. DNA manipulations (cloning, sequencing, PCR analysis, etc.) were carried out by standard procedures (Sambrook et al., 1989).

Results

Generation of chromosomal deletion for *pros54* by *P*-element-induced male recombination

Pros54 is located in the cytological region 78E on the third chromosome; the transcription start site is at 21376284 bp (GadFly). By Southern blotting, we screened a series of *P*-element insertions, mapped to this region, from a large-scale insertional mutagenesis experiment on the third chromosome (Deák et al., 1997). The *P-lacW* insert in line 0554/18 was found near the 3′ end of *pros54*. Sequencing of the *P*-element flanking regions cloned in rescue plasmids revealed that the insertion point was in the annotated genes *CG7181* and *Vha M9.7-2* (CG7625), 937 bp downstream of the stop codon of *pros54* (Fig. 1A). The insertion line 0554/18 was originally classified as pharate-adult/adult semilethal (Deák et al., 1997). We found that the homozygotes exhibited a delayed development (a 17 day generation time) and a relatively low viability. However, the adult homozygotes proved to be fertile, so it was possible to establish a homozygous stock.

To obtain a null allele of *pros54*, we isolated a series of chromosomal deletions generated by *P*-element-induced male recombination as described previously (Preston et al., 1996). Males carrying the *Δ2-3* transposase source on the second chromosome and the 0554/18 *P(w*$^{+}$*)* insertion at 78E on the third chromosome over two dominant selectable markers (*Gl* to the left at 70C, and *Sb* to the right at 89B) were crossed to *w*^{1118} homozygous females. From the offspring, the *Gl P(w*$^{+}$*)* + and the + *P(w*$^{+}$*) Sb* recombinants were selected as single males and crossed to females carrying balancers for the third chromosome to establish lines. After a cross was found to be fertile, the male was separated for DNA preparation.

Genomic DNAs were then screened for deletions to the left of the *P*-element by PCR analysis with primer E located 380 bp upstream of the *pros54* transcription start site and primer PIR located in the *P*-element inverted repeat (Fig. 1A). This primer pair gives a PCR product of 2.7 kb on the DNA of the original line 0554/18 (data not shown). Among 30 recombinant lines showing the *Gl P(w*$^{+}$*)* + phenotype, we found three that gave a PCR product shorter than ~2700 bp, suggesting a deletion toward the left side of the *P*-element. In one of these recombinants, the PCR product was ~600 bp in length, indicating a ~2100 bp long deletion between the primer pair. Sequencing of the PCR product revealed that the exact size of the deletion was 2095 bp (Fig. 1B), and the deletion eliminated the whole of the coding region of the annotated gene *CG7181* and the 5′ regulatory region and the first exon of gene *Vha M9.7-2* (CG7625), together with more than 90% of the coding region of *pros54*. This means that in the deletion line, the 5′ end of *pros54*, including the regulatory region, the first exon and intron and a short segment of the second exon, were retained; altogether, these code for the first 29 amino acids of

the p54 protein. Western blot analysis (see later) revealed that the homozygous deletion *Df(3L)pros54P(w$^+$)* did not produce any detectable p54 protein and therefore could be considered to be a null allele.

To remove *Gl* and any possible background mutation by recombination, we crossed the *Df(3L)pros54P(w$^+$)* line to *w^{1118}* flies, and the 'purified' *Df(3L)pros54P(w$^+$)* chromosome was balanced over *TM3, Sb* on a *w^{1118}* background. Deletion homozygotes of this line displayed lethality during the first and second larval stages.

Rescuing the functions of genes *CG7181* and *Vha M9.7-2* (CG7625) removed by the *Df(3L)pros54P(w$^+$)* deficiency

In order to examine the real phenotype of the null allele of *pros54* alone, the other two genes affected by the deletion *Df(3L)pros54P(w$^+$)* should be rescued.

First, an 8 kb *Hin*dIII fragment derived from a 15 kb genomic clone (Haracska and Udvardy, 1995) that overlapped all three genes (Fig. 1C) was cloned into the transforming vector *pP(CaSpeR-4)* and used for transformation (*Hin*dIII rescue construct). The flies that were homozygous for *Df(3L)pros54P(w$^+$)* and carried one copy of the *Hin*dIII rescue construct were fully viable and fertile. This proved that (i) the *Hin*dIII rescue construct is able to rescue all three genes, and (ii) the deficiency chromosome has no other background mutation.

Next, a 2.8 kb long genomic *Sac*I fragment overlapping the *pros54* gene (Fig. 1C) was cloned into the *Pst*I site of the *pP(W8)* transforming vector (*Sac*I rescue construct). The animals that were homozygous for the *Df(3L)pros54P(w$^+$)* deficiency and carried one copy of the *Sac*I rescue construct were early larval lethals, and none of them developed beyond the second larval stage. This means that the *Sac*I rescue construct did not contain all the genetic information necessary for rescuing the *Df(3L)pros54P(w$^+$)* deficiency.

Finally a 2.7 kb long *Pst*I fragment of the same genomic clone overlapping the entire *CG7181* and *Vha M9.7-2* (CG7625) genes and a short 3′ segment of *pros54* (Fig. 1C) was also inserted into *pP(CaSpeR-4)*. For injection into *Drosophila w^{1118}* embryos, we used a construct (named hereafter the *Pst*I rescue construct) in which the region coding for the C-terminal 116 amino acids of p54 has an orientation opposite to that of the *mini-w$^+$* marker gene. Because of the lack of appropriate transcription and translation regulatory sequences, this construct cannot support the production of a truncated C-terminal p54 protein product. As all these constructs were made of genomic fragments, they contained the authentic regulatory sequences allowing correct spatial and temporal expression of the genes.

Homozygous flies for the *Df(3L)pros54P(w$^+$)* deficiency that carried one copy of the *Pst*I rescue construct and one copy of the *Sac*I rescue construct were fully viable and fertile. This showed that the *Pst*I and the *Sac*I rescue constructs together contained all the genetic information removed by the *Df(3L)pros54P(w$^+$)* deficiency. When the *Pst*I rescue construct was in combination with the homozygous *Df(3L)pros54P(w$^+$)*, the animals exhibited larval-pupal lethality (see later). This combination [*yw; Pst/y$^+$CyO; Df(3L)pros54P(w$^+$)/ Df(3L)pros54P(w$^+$)*] lacked *pros54* but had full copies of the *CG7181* and *Vha M9.7-2* (CG7625) genes. Therefore, the larval-pupal lethality was a consequence of the deletion of *pros54* alone and represents the p54-null phenotype. From here on, this combination will be denoted *Δp54*.

Table 1. Mutant mortality during development

Developmental stage	Mortality (%)
Embryo	0
L1 larval	40
L2 larval	20
L3 larval	13
Pupa	27

Deletion of *pros54* results in larval-pupal polyphasic lethality

Δp54 mutant animals display polyphasic lethality during their development. In the embryonic phase, mortality is apparently similar to that in the control, that is, most of the embryos hatched as first instar (L1) larvae (data not shown). During larval development, there is an increase in mortality (Table 1). The surviving larvae develop more slowly than the wildtype, and the majority of them reach maturity and pupariate 3-4 days later than their heterozygous siblings. Although the size of the mutant larvae is almost normal, some of their internal organs are significantly smaller, especially in the late-pupariating ones. For example the brain and the ventral ganglion are about half of the normal size (Fig. 2G). Only 27% of the hatched larvae survive up to the end of larval development and form puparia. These mutant puparia are smaller than the wild-type ones and have a characteristic bent shape (Fig. 2A,B). The cuticle of the mutant puparia is softer, lighter in colour and not so rigid as the wild-type one, suggesting incomplete tanning of the mutant cuticle. In about one-third of the puparia, a complete pupal cuticle is secreted, which fully covers the head, thorax and abdomen. However, the head eversion is mostly incomplete and the appendages (wings and legs) are always smaller and shorter than normal (Fig. 2D). *Δp54* mutant pupae never developed beyond this stage. In half of the puparia, only imaginal disc derivatives (head, thorax, adult appendages and the region of the external genitalia) secrete the pupal cuticle. In the later examples, the pupal cuticle is either missing or incomplete on the abdomen (Fig. 2B). In extreme cases (10% of the specimens), some pupal cuticles can only be found in the regions of the head and the external genitalia (Fig. 2F). All the mutant puparia dry out in 1-2 days, suggesting that the puparial cuticle can not prevent desiccation, unlike the situation in the wildtype. We successfully prevented desiccation by keeping the mutant puparia in a wet environment. Even if they remained alive for a longer time, they could not develop further and they finally died. For example, the mutant specimens in Fig. 2A,B,D,F were kept in a wet chamber for 60 hours before the pictures were taken.

Histolysis of the larval tissues (salivary gland, midgut epithelium, body wall muscles, etc.) apparently occurs, and the fat body breaks up into single cells (Fig. 2B,F). The metamorphosis seems to stop at this point because the inner organs of the adult are not formed and the secretion of an adult cuticle with hairs and bristles never takes place. It should be noted that limited cell proliferation could sometimes be

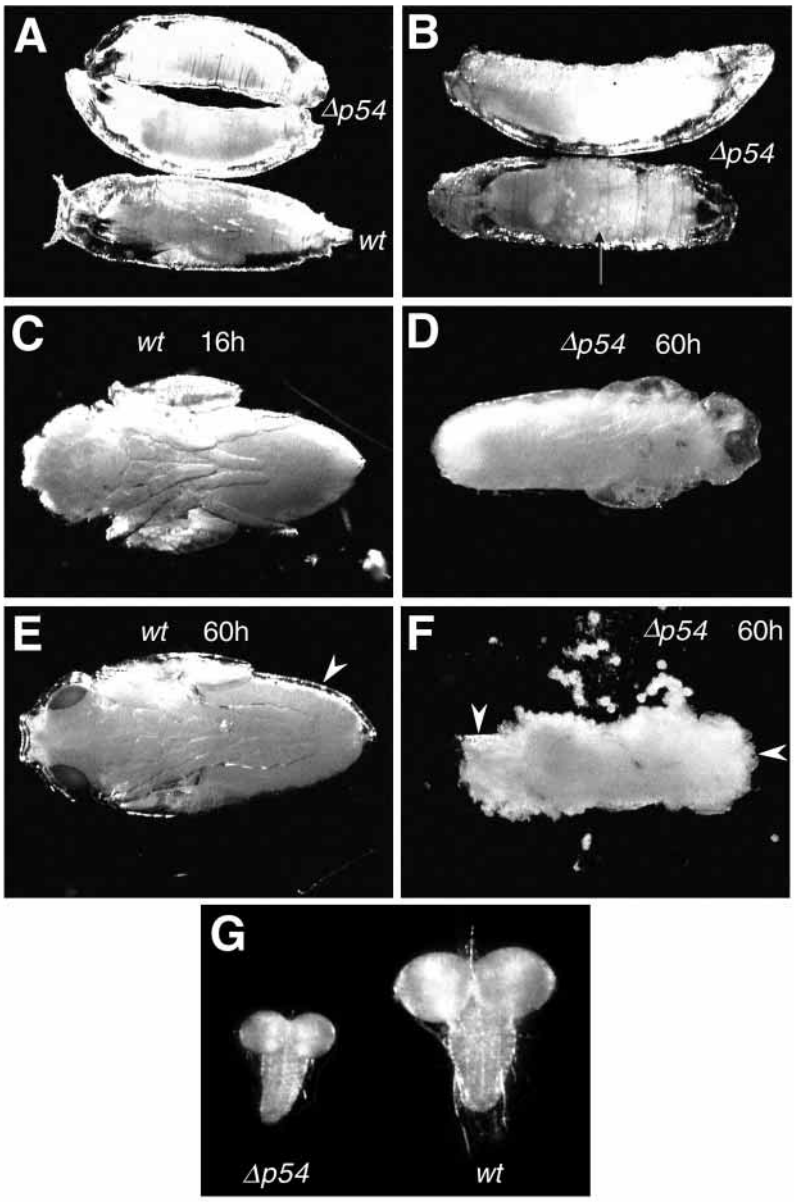

Fig. 2. Pupal lethal phenotype of *Δp54* mutant. (A) 60-hour-old (APF) puparia of *Δp54* (top and middle) and Oregon R wild-type (bottom). The mutant puparia are characteristically bent and smaller than the wild-type. (B) 60-hour-old (APF) puparia of *Δp54* (top: side view, bottom: dorsal view). The pupae inside are separated from the puparial cuticle (see also A). In the bottom animal the pupal cuticle was laid down in the head and tail regions but remained open in the middle of the body and the internal tissues are exposed (arrow). (C-F) Pupae removed from the puparial case for comparison. (C) In the 16-hour-old (APF) wild-type pupa the main body parts of the adult (head, thorax, abdomen) are formed and the wings and legs everted. (D) In the 60-hour-old mutant pupa the head and thorax are significantly smaller and the appendages shorter than those of the 16-hour-old wildtype. Adult cuticle secretion and eye pigment deposition were never observed. (E) 60-hour-old wild-type pupa. The hypoderm already separated from the pupal cuticle (arrowhead) in preparation for the adult cuticle secretion. Pigment deposition is visible in the eyes. (F) A 60-hour-old (APF) mutant pupa with minimal signs of development: the pupal cuticle can be found only in the regions of the head and the external genitalia (arrowheads). On the other parts of the body the internal tissues are exposed. D and F represent the two extremes of the *Δp54* mutant phenotype (see text for details). A,C,E are anterior to the left; B,D,F are anterior to the right. (G) Larval brain from mutant and wild-type wandering larvae.

observed in the imaginal rudiments of the midgut epithelium (imaginal islands and imaginal ring at the midgut-hindgut boundary). These tissues contain small cells with small diploid nuclei that could easily be distinguished from the large polythenic larval cells by DAPI staining (data not shown).

Deletion of *pros54* results in multiple mitotic defects

The activity of the proteasome is essential for normal cell cycle progression. To determine the role or contribution of subunit p54 to the overall function of the proteasome in the cell cycle, we analysed neuroblast preparations from larvae lacking this subunit.

The examination of mitotic cells in squashed preparations of the central nervous system from *Δp54* third instar larvae revealed several characteristic features. First, the mitotic index in *Δp54* preparations is increased compared with that in the wildtype (Table 2). The frequency of prometaphase and metaphase forms is also higher in the mutant. Additionally, a significant proportion of *Δp54* mitotic cells have over-condensed chromosomes (Fig. 3B), similar to those caused by colchicine treatment. These features arose as a 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. Moreover, a significant proportion of the cells in prometaphase and metaphase show no obvious centromeric connection between at least some of the sister chromatides (Fig. 3F), which indicates premature sister chromatid separation. Some of the cells in anaphase display chromosome bridges and lagging chromosomes. Characteristically, in about 19% of mitotic cells all major chromosomes are arranged in a circle with the centromeres pointing toward the centre, and the dot-like fourth chromosomes are always located in the middle of the circle (Fig. 3C). These circular mitotic figures (CMFs) are similar to CMFs found in *mgr*, *polo* and *aur* mutants in *Drosophila*

Table 2. Mitotic phenotypes of larval CNS cells in *Δp54* mutant and wild-type larvae

Genotype	Oregon R	*Δp54*
Number of metaphase figures*	2788	2030
Number of anaphase and telophase figures*	1237	476
Overcondensed chromosomes†	0.8	24.6
Circular mitotic figures (%)†	0	18.6
Early chromosome separation (%)†	2.4	15.2
Aneuploid or polyploid figures (%)†	0	6.0
M:A ratio‡	2.2	4.3
Mitotic index§	1.7	4.1

*Total number of mitotic figures scored from six preparations for *Δp54* and from five preparations for *Oregon R*. The larger number of mitotic figures in the *Oregon R* strain is due to the much larger brain size.

†This is the percentage of mitotic cells and not that of the total number of cells.

‡Metaphase:anaphase ratio

§The mitotic index was determined after scoring the total number of cells and mitotic figures in 15-20 fields/preps from five preparations for both genotypes.

(Gonzalez et al., 1988; Sunkel and Glover, 1988; Glover et al., 1995), where it was suggested that they are caused by monopolar spindles. Monopolar spindles are formed as a consequence of failure(s) in centrosome duplication and/or separation. A further characteristic feature of *Δp54* mutants is the high frequency of aneuploid (Fig. 3B,E) and polyploid (Fig. 3D) cells. The existence of tetra- and octaploid cells suggests that they were able to escape mitotic arrest and undergo further cell cycle(s) without chromosome segregation or cytokinesis. The frequency of all these abnormal mitotic figures is summarized in Table 2.

Molecular analysis of the 26S proteasome present in the *Δp54* pupae

An immunoblot assay with subunit-specific monoclonal antibodies unequivocally proves the complete loss of subunit p54 in *Δp54* pupae. In a total protein extract prepared from a single 24-hour-old wild-type pupa, all four subunits, including p54, can be detected in an immunoblot assay by using four different subunit-specific monoclonal antibodies (Fig. 4, lane 1). This is in sharp contrast to the immunoblot pattern obtained on the *Δp54* pupal protein extract (lane 2), in which the p54 subunit was not detected. The absence of p54 protein in the deletion mutant is in agreement with the results of PCR amplification experiments performed with different primer pairs and the genomic DNA of strains carrying different rescue constructs (data not shown).

We have demonstrated previously that the 26S proteasome is present at a very high concentration in *Drosophila* embryos, and its concentration declines during the larval stages of development (Udvardy, 1993). The high concentration of the 26S proteasome in 0- to 2-hour-old embryos indicates that its deposition is due to a maternal effect, and the maternally stored proteasome particles are gradually depleted during the larval developmental stages. The developmental profile of the 26S proteasome during the pupal stage, however, has not been tested previously. The abundance of the 26S proteasome was followed by an immunoblot assay during the embryonic-larval-pupal developmental stages of the Oregon R wild-type *Drosophila* strain. Protein extracts were prepared from a single third instar larva, a single pupa of different ages and 0- to 12-hour-old embryos. The protein extracts were fractionated by SDS-PAGE (9% gel) and immunoblotted with a mixture of two monoclonal antibodies specific for subunits p54 and p48A. The embryonic extract was prepared from 1 mg of embryo, which is the average weight of a third instar larva or a pupa. As shown in Fig. 5, the concentration of the 26S proteasome is very high in the embryos; it is very low in the third instar larvae (after a short exposure, it is not detected in a single third instar larva, but it can be detected after a longer exposure; data not shown), and its concentration increases sharply during the first 4 hours of pupal development. This sudden increase in the 26S proteasome concentration may be essential to support the sharp increase in mitotic activities of imaginal discs during the larval-pupal developmental transition. The increased demands of the proteasomal activity and the compromised function of the mutant 26S proteasome may be the reasons for the observed lethality.

Immunoblot analysis with an anti-ubiquitin antibody revealed that there is no significant increase in the total amount of multiubiquitinated proteins in the deletion mutant. However, there was a shift in the proportion of highly multiubiquitinated proteins in the

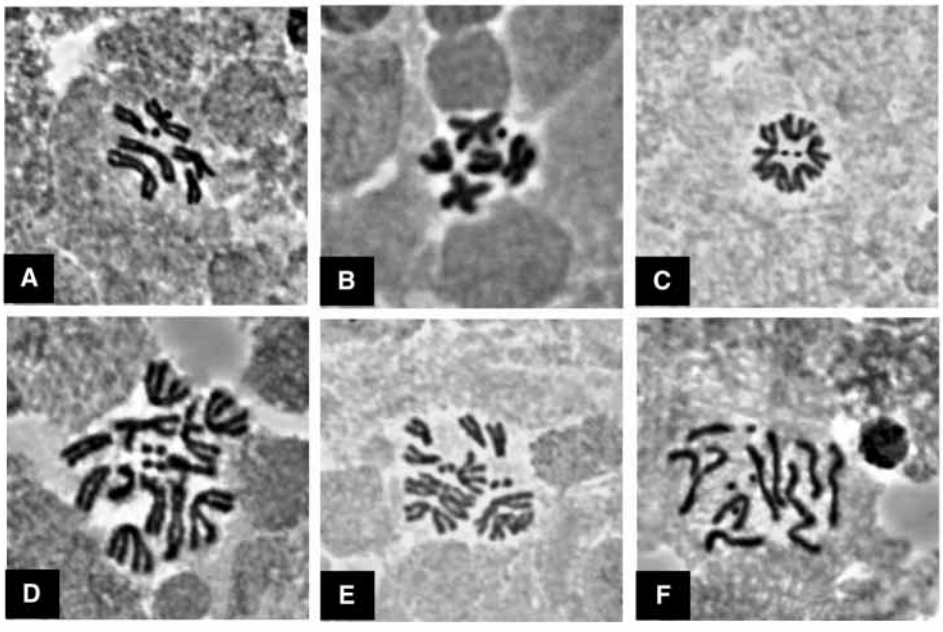

Fig. 3. Mitotic figures from wild-type and *Δp54* mutant larval brains. Aceto-orcein-stained metaphase figures from wild-type (A) and *Δp54* (B-F) third instar larval brains. *Δp54* mitotic cells frequently show highly condensed chromosomes (B), aneuploid (B,E) or polyploid (D) chromosome sets and circular mitotic figures (C). Characteristically, some cells appear to prematurely separate their sister chromatids (F).

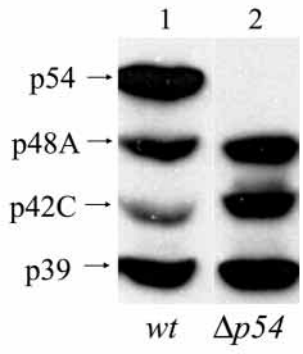

Fig. 4. Complete lack of subunit S5a/Rpn10/p54 in *Δp54* mutant pupae. Total protein extracts of wild-type (lane 1) and *Δp54* (lane 2) pupae were fractionated on 8% SDS-PAGE and immunoblotted with a mixture of four different monoclonal antibodies specific for subunits p54, p48A, p42C and p39.

Fig. 5. Developmental profile of the 26S proteasome in the Oregon R wild-type strain. Total protein extract from 0- to 24-hour-old embryos (lane 1), 3rd instar larvae (lane 2), 0-hour-old (lane 3), 2-hour-old (lane 4), 4-hour-old (lane 5), 6-hour-old (lane 6), 8-hour-old (lane 7), 10-hour-old (lane 8) prepupae as well as 12-hour-old (lane 9), 18-hour-old (lane 10) and 24-hour-old (lane 11) pupae were fractionated on 8% SDS-PAGE and immunoblotted with a mixture of two different monoclonal antibodies specific for subunits p54 and p48A. The age of the specimens is given in hours after white puparium formation.

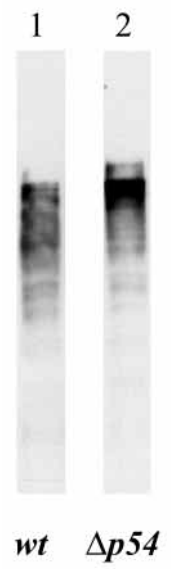

Fig. 6. Multiubiquitinated protein profile in wild-type and *Δp54* pupae. Total protein extracts prepared from a single wild-type (lane 1) or a single *Δp54* (lane 2) pupa (20 hours APF) were fractionated on 9% SDS-PAGE and immunoblotted with a polyclonal anti-ubiquitin antibody.

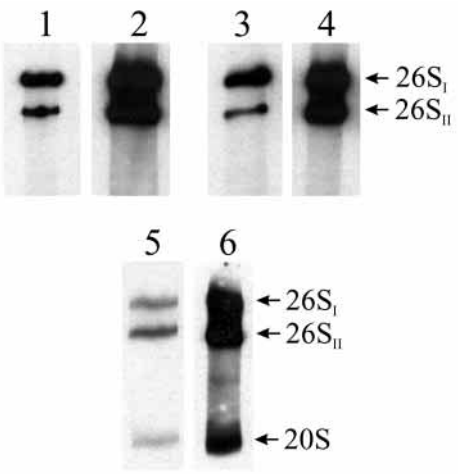

Fig. 7. 26S proteasomes in wild-type and *Δp54* pupae analysed by native polyacrylamide gel electrophoresis. Total protein extracts prepared from wild-type (lanes 1, 3 and 5) or *Δp54* (lanes 2, 4 and 6) pupae (20 hours APF) were fractionated on native polyacrylamide gel and immunoblotted with a monoclonal antibody specific for subunit p42C present in the base subcomplex (lanes 1 and 2), with a monoclonal antibody specific for a subunit p39 present in the lid subcomplex (lanes 3 and 4) or with a polyclonal antibody specific for the 20S proteasome, the catalytic core (lanes 5 and 6).

pupae of the deletion mutant (Fig. 6). This shift may be a consequence of either an upregulation of the enzyme cascade responsible for the multiubiquitination of proteins or a downregulation of the deubiquitinating enzyme activity in the mutant cells. It is more probable, however, that the accumulation of highly multiubiquitinated proteins is the manifestation of an impaired degradation of a certain class of multiubiquitinated substrate proteins.

In *Saccharomyces cerevisiae*, deletion of the S5a/Rpn10/p54 subunit destabilises the RC of the 26S proteasome, which comes apart into lid and base subcomplexes, during the purification procedure (Glickman et al., 1998b). To test the stability of the mutant *Drosophila* RC, freshly prepared protein extracts from 4-to 24-hour-old wild-type or mutant pupae were fractionated on a native polyacrylamide gel, and the integrity of the 26S proteasome was analysed by an immunoblot technique. It is known that the intact 26S proteasomes can be resolved into two distinct isoforms by native PAGE. These isoforms probably correspond to the singly capped and the doubly capped forms of the enzyme seen in the electron microscope (Glickman et al., 1998a; Hölzl et al., 2000). The electrophoretic pattern of the mutant 26S proteasome was indistinguishable from that of the wild-type enzyme (Fig. 7). Both in the wild-type and in the mutant pupal extracts, monoclonal antibodies specific for subunits of either the lid or the base subcomplexes stained both isoforms with equal intensity, indicating that in these bands complete RCs and not lid or base subcomplexes are present. Furthermore, the bands that are recognised by the lid- and the base-subcomplex-specific monoclonal antibodies also reacted with a polyclonal antibody specific for the catalytic core, indicating that both bands correspond to the 26S proteasome. Thus, deletion of subunit p54 does not destabilize the RC and does not interfere with the assembly of the RC and the catalytic core in *Drosophila*. The electrophoretic mobilities of the 26S proteasome isoforms obtained from wild-type or *Δp54* strains are very similar. Because of the high resolving power of native PAGE, this indicates that the absence of subunit p54 does not induce a structural rearrangement in the 26S proteasome extensive enough to influence the electrophoretic mobility of the particle. Free RC [running between the $26S_{II}$ and the 20S proteasome (Hölzl et al., 2000)] is not present at a detectable level in the mutant pupae. Although native PAGE is only an analytical method, with obvious limitations in the sensitivity of detection, the lack of immunoreactive material in the lower

part of the gel indicates that neither free subunits nor partially assembled particles are present in significant quantity in mutant pupae.

The increased demands of the proteasomal activity during the pupal developmental stage, and the compromised function of the mutant 26S proteasome in *Δp54*, allowed the study of a specific aspect of the regulation of expression of the genes encoding proteasomal subunits. Assuming a feedback regulatory circuit, in which increased proteasomal activity demands induce the upregulation of the expression of genes coding for proteasomal subunits, one would expect a higher 26S proteasome content in the mutants, or at least the upregulation of those subunits involved in the coordinated feedback regulation. To compare the 26S proteasome contents of the wild-type and *Δp54* pupae, single 24-hour-old pupae from both strains were disrupted directly in SDS sample buffer and analysed by the immunoblot technique with polyclonal antibodies raised against either the highly purified RC or the highly purified 20S proteasome. As shown in Fig. 8, there are huge differences in both the RC and the 20S proteasome content of the wild-type and *Δp54* pupae. Densitometric analysis revealed that the RC contents of the *Δp54* pupae is at least 20-fold higher than that of the wildtype. This difference is not due to an unequal loading of the proteins, because no difference in immunoblot intensities was found when antibodies specific for two different household proteins were used on the same filter (Fig. 8, lanes 5-8). It was even more surprising that both the RC and the catalytic core of the 26S proteasome exhibited an extreme upregulation in the deletion mutants (Fig. 8, lanes 1-4).

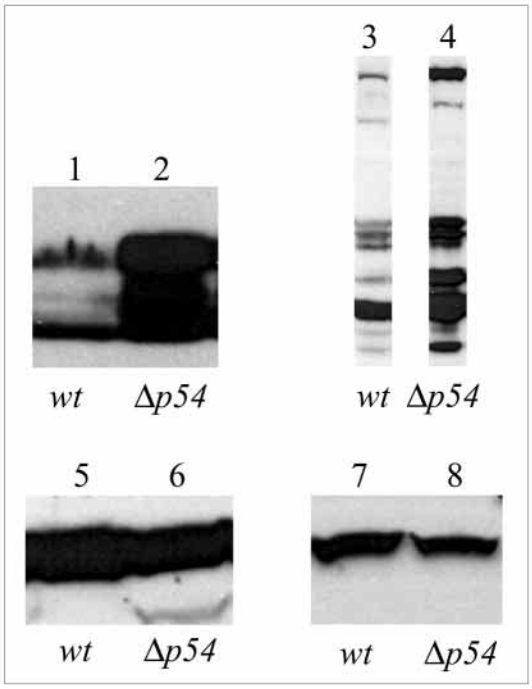

Fig. 8. Accumulation of proteasomal proteins in *Δp54* animals. Total protein extracts prepared from a single wild-type (lane 1) or a single *Δp54* (lane 2) pupa (20 hours APF) were fractionated on 10% SDS-PAGE and immunoblotted with a polyclonal antibody specific for the catalytic core. The same extracts fractionated on a 8% SDS-PAGE were immunoblotted with the following polyclonal antibodies: anti-regulatory complex antibody (lanes 3 and 4), anti-glycogen phosphorylase antibody (lanes 5 and 6) and anti-karyopherin β antibody (lanes 7 and 8).

Discussion

The first step in the catalytic cycle of the 26S proteasome is substrate selection, that is, recognition and binding of multiubiquitinated proteins. Discovery of a RC subunit that can selectively recognise and bind the multiubiquitin chains in an in vitro overlay assay was the first step towards elucidation of the mechanism of substrate recognition (Deveraux et al., 1994). The discovery that deletion of this gene does not influence the viability of yeast cells (van Nocker et al., 1996b) was unexpected, since it was thought that substrate recognition is an essential step in the degradation process. This observation suggested that substrate recognition is a more complicated process, probably involving several different, partially overlapping mechanisms.

The discovery that the DNA repair protein Rad 23 carries an N-terminal ubiquitin-like domain (UBL) that interacts with the 26S proteasome initiated an alternative approach to the understanding of the selective substrate recognition mechanism of the 26S proteasome (Watkins et al., 1993; Schauber et al., 1998; Hiyama et al., 1999). Besides the UBL domain, Rad 23 contains two partially homologous sequence motifs, the ubiquitin-associated domains (UBA), present in several cellular proteins, which can recognise and bind ubiquitin moieties (Hofmann and Bucher, 1996; van der Spek et al., 1996; Chen et al., 2001). Originally, monoubiquitin was considered to be the interacting partner of the UBA domain (Bertolaet et al., 2001), but more recent data indicate that the multiubiquitin chains are bound preferentially (Wilkinson et al., 2001; Rao and Sastry, 2002). It is believed that proteins carrying both UBL and UBA domains are involved in the substrate selection for the proteasome. The UBA domain is required for the selective recognition and binding of multiubiquitinated proteins, whereas the UBL domain generates the interaction with the 26S proteasome, a prerequisite for presenting the multiubiquitinated substrate proteins for degradation. This assumption is supported by the observation that not only Rhp 23 (the fission-yeast homologue of Rad 23) but also another fission yeast protein, Dph1, which carries both UBA and UBL domains, has the same dual properties: it can specifically recognise and bind the multiubiquitin chains and interact with the 26S proteasome (Wilkinson et al., 2001). The UBL domain of Rad 23 interacts with the S5a/Rpn10/p54 subunit of the RC (Hiyama et al., 1999). The coordinated role of the UBA-UBL-containing proteins and the S5a/Rpn10/p54 subunit in substrate selection is supported by the observation that the single and double deletion mutants of Rhp 23, Dhp1 and Pus 1 (the fission-yeast orthologue of S5a/Rpn10/p54) are viable, whereas triple deletion of these genes was lethal (Wilkinson et al., 2001). Thus, UBA-UBL-containing proteins in co-operation with S5a/Rpn10/p54 are indispensable for the degradation of essential proteins, and the stabilisation of these proteins is lethal for the cell.

Nevertheless, the role of S5a/Rpn10/p54 in substrate selection is still controversial. If its ubiquitin-binding function is required only for the targeting of UBL-containing proteins

to the proteasome, why does it show strict preference for multiubiquitin chains in an in vitro ubiquitin-binding assay, although there is only one single ubiquitin moiety in the UBL domain? Furthermore, if it is assumed that UBA-containing proteins are the true multiubiquitin chain receptors, and the only role of S5a/Rpn10/p54 in substrate selection is its ability to interact with the UBL domain of UBA-containing proteins, why is the yeast S5a/Rpn10/p54 deletion mutant viable? It is more reasonable to suppose that there are structurally distinct classes of multiubiquitinated proteins, which are recognised and targeted for degradation by distinct but partially overlapping mechanisms. Certain multiubiquitinated proteins may be selected and targeted exclusively by the Rpn10/S5a subunit. For the recognition of other classes of proteasome substrates, UBA-containing receptors are required. Targeting of these substrates may require an UBL domain in the receptor, which alone or in cooperation with unmasked ubiquitin moieties of the multiubiquitin chain may interact with the S5a/Rpn10/p54 subunit, promoting the targeting of the substrate to the proteasome.

To explain the viable phenotype of the yeast S5a/Rpn10/p54 deletion mutant, alternative substrate recognition and targeting mechanisms must be considered. Database analyses have identified eight proteins with a UBA domain in the fission yeast genome (Wilkinson et al., 2001). All eight UBA proteins are able to bind multiubiquitin chains, but only two of them contain an additional UBL domain. The interactions of UBA proteins (without an additional UBL domain) with the 26S proteasome have never been tested. It may be assumed that these UBA proteins may target multiubiquitinated proteins to the proteasome by interacting with RC subunits other than the S5a/Rpn10/p54; this is a plausible alternative, which may explain the viable phenotype of the yeast S5a/Rpn10/p54 deletion mutant. This assumption is supported by the observation that, although Pus 1 is not required for cell viability in the fission yeast, deletion of Pus 1 is synthetically lethal with mutations of three other RC subunits (Rpn12, Rpn11 and Rpn1). Overexpression of the wild-type Pus 1 protein, but not its mutant version without multiubiquitin-binding activity, could rescue a temperature-sensitive mutation of Rpn12 (Wilkinson et al., 2000). Moreover, the close physical association of Pus 1 and Rpn12 proteins has been demonstrated, suggesting their cooperation in substrate selection.

The hypothesis of a direct and unaided role of S5a/Rpn10/p54 as a multiubiquitin receptor in yeast is supported by the observation that the degradation of certain proteasome substrates is impaired in the yeast Df(S5a/Rpn10/p54) mutant (van Nocker et al., 1996b). This observation supports the notion that S5a/Rpn10/p54 functions 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 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 few 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 mutants suggest that proteins involved in the cell cycle regulation may belong to this specific class of substrate proteins.

The viability of *Δp54* embryos and larvae is due to a large pool of maternally stored 26S proteasomes in the embryos, which becomes only gradually depleted during the larval stage. Thus, it cannot be stated 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 indispensable but essential only in certain phases of the development. The polyphasic larval-pupal lethality of the present mutant, however, suggests that, as soon as the maternally stored wild-type 26S proteasome depot is depleted, mutant proteasomes, even in excess, can not rescue the lethality.

Recently it has been shown that in mice, Rpn10 mRNA is present in at least five distinct developmentally regulated alternatively spliced forms. Protein products of these forms are components of the 26S proteasome, with an apparently similar affinity for multiubiquitinated lysozyme (Kawahara et al., 2000). RT-PCR analysis of polyA$^+$ RNAs prepared from *Drosophila* embryos, pupae and flies revealed a single mRNA product (data not shown). Thus the pupal lethality of our mutant is not a consequence of the elimination of a pupal-specific form of the p54 mRNA, which can not be complemented with other spliced variants of the p54 mRNA.

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 owing to the lack of subunit p54.

The specific crosslinking of a reactive version of a tetraubiquitin chain to the S6′/Rpt5/p50 ATPase subunit (Lam et al., 2002) suggests the involvement of this subunit in substrate selection. This observation, however, does not exclude a similar role for other subunits in this process. The crosslinking of two polypeptides depends on the optimum spatial configuration of two reactive side-chains of the interacting polypeptides, which are specific for the applied crosslinker. If the distance between these reactive side-chains is out of the range of the spacer arm of the crosslinker, covalent crosslinking cannot occur, even between strongly interacting polypeptides. This question will ultimately be settled by the identification of cellular proteins processed selectively by the different recognition mechanisms.

In the yeast, RPN4 was identified as a transcription factor involved in the coordinated regulation of genes encoding proteasomal subunits (Mannhaupt et al., 1999; Xie and Varshavsky, 2001). RPN4 is a very short-lived protein, a substrate of the 26S proteasome, which interacts with the RC subunit Rpn2. 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 (Xie and Varshavsky, 2001). In this circuit, RPN4 upregulates the expression of genes encoding proteasome subunits, and it is finally destroyed by the assembled active proteasomes. In higher eukaryotes, the coordinated regulation of genes encoding proteasomal subunits has never been demonstrated. If a similar feedback circuit operates in higher eukaryotes, the accumulation of proteasomal

subunits would be expected under conditions when the demand for proteasomal activity increases and/or the presumed regulator of the circuit is stabilised. 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.

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