Mol Genet Genomics (2007) 278:17–29
DOI 10.1007/s00438-007-0223-3

ORIGINAL PAPER

Molecular characterization of the Rpt1/p48B ATPase subunit of the *Drosophila melanogaster* 26S proteasome

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Received: 26 September 2006 / Accepted: 11 February 2007 / Published online: 12 April 2007

Abstract The function and the molecular properties of the Rpt1/p48B ATPase subunit of the regulatory particle of the *Drosophila melanogaster* 26S proteasome have been studied by analyzing three mutant *Drosophila* stocks in which P-element insertions occurred in the 5′-non-translated region of the Rpt1/p48B gene. These P-element insertions resulted in larval lethality during the second instar larval phase. Since the Rpt1/p48B gene resides within a long intron of an annotated, but uncharacterized *Drosophila* gene (CG17985), the second instar larval lethality may be a consequence of a combined damage to two independent genes. To analyze the phenotypic effect of the mutations affecting the Rpt1/p48B gene alone, imprecise P-element excision mutants were selected. One of them, the pupal lethal P1 mutation, is a hypomorphic allele of the Rpt1/p48B gene, in which the displacement of two essential regulatory sequences of the gene occurred due to the insertion of a 32 bp residual P-element sequence. This mutation caused a 30-fold drop in the cellular concentration of the Rpt1/p48B mRNA. The decline in the cellular Rpt1/p48B protein concentration induced serious damage in the assembly of the 26S proteasomes, the accumulation of multiubiquitinated proteins, a change in the phosphorylation pattern of the subunit and depletion of this ATPase protein from the chromatin.

Keywords 26S proteasome · Regulatory particle · ATPase subunit · P-element insertion · Rpt1/p48B subunit

Communicated by G. Reuter.

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Introduction

The ubiquitin-proteasome system (UPS) is responsible for the regulation of a large variety of biological processes, such as the cell cycle, DNA repair, transcription, signal transduction, antigen presentation, garbage disposal, stress response, neurological disorders and so on (reviewed by Hochstrasser 2006). Regulation of these biological pathways is highly interconnected, and the controlled intracellular proteolysis, perhaps the most thoroughly studied function of the UPS, is the common link involved in the coordination of these biological regulatory networks. The central role of controlled intracellular proteolysis in the regulation of such a variety of essential biological processes is due to its irreversibility, resulting in a strictly unidirectional flow of the affected biological events. The ubiquitination enzyme cascade is the first main component of the UPS (reviewed by Pickart 2004). The enzymes involved in this cascade fulfill two critical functions: (1) recognition of specific cellular proteins intended for intracellular proteolysis, and (2) via the covalent attachment of ubiquitin moieties to the selected proteins, their marking for intracellular proteolysis. The 26S proteasome, the second main component of the UPS, is a large multiprotein complex which is responsible for the recognition, binding and proteolytic degradation of multiubiquitinated proteins. The 26S proteasome is assembled in an ATP-dependent reaction from two

main subcomplexes: the catalytic particle (CP or 20S proteasome) and the regulatory particles (RP). The 20S proteasome, the CP (reviewed by Groll and Hubert 2003), is a barrel-shaped particle composed of α- and β-type subunits that form seven-membered rings stacked together in the α7β7β7α7 configuration. Three nanocompartments are located inside this particle, connected to each other by a narrow central channel. The site of protein degradation is the central nanocompartment; the functions of the two peripheral nanocompartments are not fully known. The CP is a threonine protease; a single residue threonine of the β subunits, protruding into the cavity of the central nanocompartment acts as both catalytic nucleophile and primary proton acceptor. The orifices of the central channel, which are the sites of entry of substrate proteins (Wenzel and Baumeister 1995), are situated at the bases of the barrel in the *Thermoplasma acidophilum* 20S proteasome (Löwe et al. 1995). In the crystal structure of the *Saccharomyces cerevisiae* 20S proteasome, however, these orifices are missing, indicating that the channel is gated in eukaryotes (Groll et al. 1997, 2000). The central channel has a narrow diameter, and is accessible only for completely unfolded proteins. The CP is a non-specific protease, which cannot discriminate between multiubiquitinated and non-ubiquitinated proteins: mild detergent treatment, which unfolds the proteins and opens the gated central channel, activates the protease activity of the 20S proteasome, irrespective of the ubiquitinated state of the substrate protein.

The RPs are attached to one or to both bases of the CP and in *Drosophila melanogaster* are built up from 18 different subunits (Hoelzl et al. 2000). Among them, six subunits belong to the AAA-type ATPases; all the others are structurally diverse. During conventional chromatographic purification procedures, the RP may be split into base and lid subcomplexes as a result of the artificially high ionic strength (Glickman et al. 1998). The ATPase subunits, together with three non-ATPase subunits, form the base subcomplex. The lid subcomplex is composed entirely of non-ATPase subunits. The RPs perform several critical functions of the 26S proteasome, ensuring the selectivity of the 26S proteasome for multiubiquitinated proteins (reviewed by Hershko and Ciechanover 1998), unfolding the substrate proteins by their chaperone-like activity (Braun et al. 1999; Strickland et al. 2000), opening the gated channel of the CP (Koehler et al. 2001), reprocessing the ubiquitin residues of the substrate proteins (Verma et al. 2002; Yao and Cohen 2002) and feeding the substrate into the CP. The contribution of the individual RP subunits to the different steps of the proteolytic process is far from clear. Subunit Rpn10/p54 (the yeast, *Drosophila* and human RP subunit nomenclature is listed in Table 1), in cooperation with soluble, non-proteasomal proteins, is responsible for the selective recognition and binding of multiubiquitinated proteins (Deveraux et al. 1994; Van Nocker et al. 1996; Haracska and Udvardy 1997; Lam et al. 2002; Elsasser et al. 2004; Verma et al. 2004; Hartmann-Petersen and Gordon 2004). A subunit of the lid subcomplex Rpn11/p37B, which contains a novel Zn^{2+}-metalloprotease domain, is responsible for reprocessing the ubiquitin moieties of the multiubiquitinated substrate proteins. This deubiquitinating activity, which is strictly coupled to substrate degradation, is dependent on the unimpaired Zn^{2+}-isopeptidase function of the subunit (Verma et al. 2002; Yao and Cohen 2002). The roles of most of the lid subcomplex subunits are far less well known. Rpn 1 and Rpn 2, two non-ATPase subunits of the base, link the lid and base subcomplexes.

Table 1 Human and yeast homologues of the *Drosophila* regulatory complex subunits

Drosophila	Human	Yeast
p110	S1	Rpn 2
p97	S2	Rpn 1
p58	S3	Rpn 3
p56	S4	Rpt 2
p55	S5b	Rpn 5
p54	S5a	Rpn 10
p50	S6’	Rpt 5
p48A	S6	Rpt 3
p48B	S7	Rpt 1
p42A	S10	Rpn 7
p42B	S9	Rpn 6
p42C	S8	Rpt 6
p42D	S10b	Rpt 4
p39A	S11	Rpn 9
p39B	S12	Rpn 8
p37A	UCH-37	–
p37B	S13	Rpn 11
p30	S14	Rpn 12

Several steps of the proteolytic cycle are ATP-dependent. Besides the assembly of the 26S proteasome from its subcomplexes, ATP is required for substrate unfolding, opening the gated channel of the CP, and most probably feeding the substrate proteins into the central channel of the CP. Six ATPase subunits of the RP, forming a heterohexameric ring, mediate all the ATP-dependent reactions (Rubin et al. 1998). The ATPase ring stacks to the base of the external α-rings of the 20S proteasome, this configuration ensuring optimum access for ATPase subunit Rpt 2 to open the gated channel of the CP by displacing N-terminal segments of α-type subunits involved in the gating. Unfolding of the substrate protein is probably performed by concerted action of the ATPase ring. Mutagenesis studies in yeast indicate that the individual ATPase subunits execute

distinctly different functions, despite the high level of sequence similarity of the ATPase subunits (Rubin et al. 1998). Our knowledge concerning the functions of the different ATPase subunits in higher eukaryotes is even more limited. As a first step of an approach to this problem, here we analyze the structural and functional consequences of a hypomorphic mutation affecting the Rpt1/p48B ATPase subunit of the *Drosophila* 26S proteasome.

Materials and methods

P-element remobilization

Fly stocks were raised and crosses were performed at 25°C on standard Drosophila medium. To mobilize the P{lacW}Rpt1^{k11110} element, w; P{lacW}/CyO females were crossed to w; CyO, P{Δ 2–3}/Bc Gla Fr males. Single w; P{lacW}/ CyO, P{Δ 2–3} jumpstarter males were crossed to yw; CyO/Sco virgins. Single white-eyed flies lacking the P-insertion (ΔP{lacW}/CyO) were used to establish putative mutant stocks. These were tested for complementation with the original P insertion stock and non-complementing ones were kept for further analysis.

Lethal phase determinations were carried out over the CyO,GFP balancer chromosome. The P1 mutant was maintained over the T(2;3)TSTL,CyO:TM6B balancer and this stock was used in all biochemical experiments.

Rescue of the Rpt1/p48B mutant phenotype by ectopic Rpt1/p48B expression

In order to create the pCaSpeR4-Ubi-promoter-Rpt1/p48B rescue construct, the NotI-BamHI DNA fragment containing the promoter sequence of the ubiquitin gene (Ubi-p63E) was cut out from the RHXpHSS7-Up2 plasmid (Fehon et al. 1994) and inserted into the NotI and BamHI sites of pBluescript II KS by ligation. In the resulting plasmid, the NcoI site was destroyed by cutting with NcoI, filling-in with Klenow polymerase and recircularized by ligation. The pBluesript II KS-Ubi-promoter plasmid was digested with BamHI and NotI, and the resulting 2.2 kb fragment was isolated from agarose gel with the QIAquick® Gel Extraction Kit (Qiagen) and ligated into the BamHI and NotI-cleaved pCaSpeR4 vector (Thummel and Pirotta 1992). The Rpt1/p48B gene and the surrounding genomic region were PCR-amplified using w^{1118} adult fly genomic DNA as template with the Rpt1 promoter fwd (5′ TAGAA GTTAAAAGTGGCTACACATC 3′) and far Rpt1 rev (5′ TACGATCGCCTCGGACTTATCATCACTC 3′) primers. The PCR fragment was purified with the PCR Clean-up Kit (V-Gene), cut with Mph1103I and NheI and ligated into the PstI and XbaI-cleaved pCaSpeR4-Ubi-promoter plasmid. The resulting plasmid was sequenced and the nucleotide mismatches formed during the PCR reaction were repaired by exchanging restriction endonuclease fragments carrying the mismatched sequence with another one derived from a different clone with a perfect sequence.

The pCaSpeR4-Ubi-promoter-Rpt1/p48B construct and the pπ 25.7 WC helper plasmid encoding the Δ2–3 P-element transposase were injected into w^{1118} embryos and transformed flies were selected according to standard procedures (Spradling 1986). The chromosomal localization of the P-element was determined and homozygous viable lines were used for complementation.

Polymerase chain reaction

The inverse PCR was carried out with primers (Plac4 and Plac1) following the protocol described in detail at the Berkeley *Drosophila* Genome Project Resources website (http://www.bdgp.org/about/methods/inverse.pcr). The primers used in the genomic PCR screen for mutations caused by the imprecise mobilization of the P-element were: Rpt1 rev (5′ GATAAGCTTGTTGTAGGTCATGTA GCGTGGAGTGG 3′) and Rpt1 promoter fwd (5′ TAGAA GTTAAAAGTGGCTACACATC 3′). The amplification protocol was: 35 × [95°C/30 s, 60°C/1 min, 68°C/2.5 min]. The amplification protocol with the farRpt1fwd (5′ TCGT GGGCTGCTGCTTAGAACAAAC 3′) and farRpt1rev (5′ T ACGATCGCCTCGGACTTATCATCACTC 3′) primers was 35 × [95°C/30 s, 60°C/1 min, 68°C/4.5 min]. Genomic DNA was prepared according to the BDGP protocol given above. The PCR was performed with GoTaq™ DNA polymerase (Promega).

Semiquantitative reverse transcription-coupled PCR

Total RNA was isolated from pupae and larvae with the Trizol™ reagent (Invitrogen). After DNase treatment with RQ1 RNase-Free DNase (Promega), samples were subjected to reverse transcription using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers. To compare the relative quantities of RPL23 cDNA in the samples, PCR was performed with different amounts of wild-type and mutant cDNA using RPL23 ribosomal protein specific primers (RPL23 upper: 5′ GTGATGAACTGTGCCGACAA 3′ and RPL23 lower: 5′ CCTTCATTTCGCCCTTGTTG 3′), GoTaq™ DNA polymerase and 20 cycles of amplification [95°C/30 s, 60°C/30 s, 68°C/30 s]. PCR products were detected in agarose gel and cDNA quantities giving the same band density among the mutant and wild-type samples were used to amplify the Rpt1/p48B and Rad23B cDNAs with the primers listed for the real-time PCR. After 35 cycles of amplification, products were run on agarose gel and photographed.

Real-time PCR

Primers: Rad23realtfwd (5′ ACGGGATTCTGATTACA AAAGGT 3′), Rad23realtrev (5′ CTAGACGAATCGCG TGTCAA 3′), Rpt1realtrev (5′ CTTGTCCTCCTTCTCA TCGTGCTTCA 3′), Rpt1 cDNS fwd (5′ TTCTCCCATC TCTATGCCAAATTGTTCAGTC 3′). For the quantitative determination of Rpt1/p48B, Rad23B and rosy mRNAs, total RNAs were isolated with RNeasy Kit (Qiagen) from wild-type and P1 mutant pupae according to the manufacturer's instructions. First-strand cDNA was synthetized from 1 μg RNA using TaqMan Reverse Transcription Reagent (ABI). The relative abundances of Rad23B, Rpt1/p48B and rosy mRNAs were quantified by real-time quantitative RT-PCR (ABI Prism 7300) using primers specific for the respective cDNAs and for 18S rRNA as a control, following the incorporation of SYBRGreen. C_T values were set against a calibration curve ranging over two orders of magnitude. The $\Delta\Delta C_T$ method was used for the calculation of the relative abundances (Winer et al. 1999). Since Rpt1/p48B is an intronless gene, the lack of genomic DNA contamination of the total RNA preparations was checked by analyzing the melting curves of the rosy mRNA PCRs.

Protein sample preparation

Pupae and larvae were homogenized in proteasome buffer (20 mM Tris–HCl pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% glycerol) in a micro Potter homogenizer. Samples were centrifuged at 12,500 rpm 4°C, for 15 min. To remove the floating lipoprotein contaminations, the supernatants were filtered through sterile glasswool at 4°C by mild centrifugation. The native gel electrophoresis of the proteasome particles, the immunoblotting technique and the polyclonal and monoclonal antibodies used for immunoblotting analysis were described in detail earlier (Kiss et al. 2005). The polyclonal the anti-cylin A and anti-cyline B antibodies were raised in rabbit by using bacterially expressed proteins. These antibodies recognized a single band in immunoblot assays on total pupal protein extracts, and in immunofluorescent staining exhibited the characteristic pattern: enriched around the spindle of the mitotic cells in the larval brain (manuscript in preparation). Anti-Ub antibody was obtained from DAKO. Dephosphorylation by potato acid phosphatase (Calbiochem) was performed in 50 mM PIPES pH 5.8, 1 mM DTT.

2D gel-electrophoresis

Cytoplasmic protein extracts were prepared from w^{1118} and P1 mutant pupae using the Bio-Rad ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear). Cytoplasmic extracts were further purified with the ReadyPrep™ 2D Cleanup Kit. Precipitated proteins were resuspended in a strongly chaotropic 2D rehydration buffer [7 M urea, 2 M thiourea, 4%(w/v) CHAPS, 50 mM DTT, 2%(w/v) IPG buffer pH 4–7, and 0.002%(w/v) bromophenol blue]. Samples were loaded onto Immobiline™ DryStrip gels (pH 4–7, 13 cm) (Amersham Biosciences) by rehydration loading. A multiphor™ II flat-bed electrophoresis unit (Pharmacia Biotech) was used for the first dimension isoelectric focusing (total 21 kVh run), and a 7% vertical SDS-PAGE gel for the second dimension.

Chromosome immunostaining

Chromosome spreads obtained from the salivary glands of wandering larvae were fixed in 3.7% formaldehyde dissolved in phosphate-buffered saline (PBS) followed by an incubation in 45% acetic acid. Slides were blocked in PBST (PBS+ 0.1% Tween-20) + 5% foetal calf serum for 1 h at 25°C. Slides were incubated overnight at 4°C in a mixture of anti-Rpt1/p48B polyclonal and anti-RNA polymerase II monoclonal antibodies diluted in blocking solution. Samples were washed three times for 5 min in PBST and incubated with a mixture of secondary antibodies (Alexa Fluor 555-conjugated anti-rabbit-, and FITC-conjugated anti-mouse IgGs, Dako) for 1 h at 25°C. The slides were washed again three times for 5 min in PBST, covered with VectaShield mounting medium containing DAPI, and examined with an OLYMPUS BX51 microscope. Photos were taken with an Olympus DP70 camera.

Results

Three P-element insertion mutants (Fly Base $Rpt1^{05643}$, $Rpt1^{k1110}$ and l(2)SH0675) encoding the Rpt1/p48B RC ATPase subunit of the *Drosophila* 26S proteasome were obtained from the Bloomington and Szeged Stock Centers. The sites of P-element insertions were determined by sequencing the inverse PCR fragments amplified from the genomic DNA of the respective stocks. For this purpose, the genomic DNA was cleaved with Hin6I restriction endonuclease, the DNA fragments were circularized by ligation and PCR amplification was carried out with primers derived from the 5′-terminal segment of the P-element. In all three strains the site of P-element insertion was exactly the same: between bp 31 and 32 in the 5′-non-translated region of the gene. In strains $Rpt1^{k1110}$ and l(2)SH0675, which were generated independently (obtained from the Bloomington and Szeged Stock Centers, respectively), the P{lacW} P-element was inserted at the same position, but in the opposite orientation. Thus, this site of the 5′-non-translated region of the gene is probably a hot spot of P-element insertion. Animals homozygous for $Rpt1^{05643}$ and $Rpt1^{k1110}$ P-element

insertions showed second instar larval lethality, while the lethal phase of the homozygous l(2)SH0675 was shifted to the pupal stage. The difference in the lethal phases of the homozygous Rpt1^{k1110} and l(2)SH0675 strains may be due to the orientation of the inserted P-element; in the strain l(2)SH0675, the promoter of the miniwhite (w^{+mC}) gene located in the 3′ half of the P-element is oriented toward the Rpt1/p48B gene ORF, which may support a certain level of Rpt1/p48B expression.

As the Rpt1/p48B gene resides inside a long intron of an annotated, but uncharacterized gene (FlyBase CG17985), the lethal phenotype of the homozygous P-element insertions may be a consequence of the disruption of the expressions of both. In an attempt to assign the lethal phenotype to the Rpt1/p48B gene, P-element mobilization was induced by crossing the Rpt1^{k11110} P-element insertion mutant with a transposase source, and flies with white eyes were selected. Sixty-one stocks with white eyes were established: 45 of them died in the L2 larval stage, 13 in pupal stage and 3 were viable as homozygous. The 61 stocks were analyzed by genomic PCR amplification to select for imprecise P-element excisions. If the imprecise P-element excision produces a short deletion, or a short segment of the P-element is left behind following the excision, the expression of the Rpt1/p48B gene may be damaged, without injury to the expression or the splicing of gene CG17985. We identified four larval lethal strains in which the genomic PCR generated a fragment 250–1,000 bp longer than that obtained on the wild-type genomic DNA. Sequence analysis revealed that the extra DNA is derived from the P-element. In one pupal lethal strain 160 bp P-element sequence was left behind following the P-element mobilization. In all the other strains, the size of the genomic PCR fragment was indistinguishable by agarose gel electrophoresis from that of the wild-type. Genomic PCR fragments from nine randomly chosen pupal lethal mutants of the above group, and the stock with 160 bp insertion, were sequenced. In two stocks precise excision of the P-element occurred, in seven stocks 24–35 bp and in the last stock 160 bp P-element sequence was left behind due to imprecise P-element excision (Table 2). The pupal lethality of the stocks with precise P-element excision may be due to a second site mutation as a result of a second P-element insertion-imprecise excision event induced by the transposase enzyme. The site and the nature of these putative second-site mutations have not been analyzed. The mutant phenotype therefore depended on the length of the inserted DNA; shorter insertions (below 160 bp) induced pupal lethality, while larval lethality was always a consequence of a longer insertion.

Three larval lethal (L15, L25 and L45) and three pupal lethal (P1, P56 and P78) mutants obtained following the mobilization of the P-element, and also the original P-element insertion stock (Rpt1^{k1110}), were crossed to the overlapping Df(2R)ED1715 and Df(2R)ED1725 deficiencies uncovering 303 kb genomic sequences around the Rpt1/p48B gene. The lack of complementation indicated that the lethal phenotype of the insertion mutants is not due to a remote second site mutation; the damage responsible for the lethality resides within the uncovered 303 kb genomic region. Transgenic rescue experiments were performed to analyze the role of the damage to the Rpt1/p48B gene in the generation of the lethality. In these rescue experiments, the enhancer-promoter region of the ubiquitin gene was ligated to a 2.1 kb genomic DNA fragment carrying the coding sequence of the Rpt1/p48B gene, and this construct was inserted into the P{pCaspeR4} P-element transformation vector. Transgenic stock was generated (w;If/CyO; P{pCaspeR4-Ub promoter-Rpt1/p48B}/TM6) and was crossed to the L15 larval lethal and P1 pupal lethal mutants and also to one of the original P-element insertion stocks (Rpt1^{k1110}). The flies that were homozygous for the P1 mutation and carried one copy of the rescue construct were fully viable and fertile, indicating that the pupal lethality of this mutation is due to the damage to the Rpt1/p48B gene and no other genomic injury is present within the 303 kb genomic segment uncovered by the Df(2R)ED1715 and Df(2R)ED1725 deficiencies. The transgene carrying the Rpt1/p48B gene, however, could not rescue the second instar larval lethality of the original P-element insertion stock (Rpt1^{k1110}), suggesting that the P-element insertion damaged the expression and/or the splicing of the uncharacterized gene CG17985, and this gene is essential. The transgene carrying the Rpt1/p48B gene could not rescue the L15 larval lethal mutation either, suggesting that the 600 bp long insertion, like the complete P-element, affects the CG17985 gene.

Table 2 Imprecise P-element excision mutants

Name of jumpout derivative stock	Length of residual P-element sequence (bp)	Lethal phase as homozygous
L15	600	L2
L25	1000	L2
L45	250	L2
L61	500	L2
P1	32	Pupa
P9	33	Pupa
P24	32	Pupa
P49	24	Pupa
P51	160	Pupa
P59	35	Pupa
P65	32	Pupa
P77	30	Pupa

Following imprecise P-element excision, a shorter (24–160 bp) or a longer (250–1,000 bp) P-element sequence is left behind at the site of P-element insertion. Although these sequences are located in the 5′-untranslated region of the gene, even the shortest one causes lethality. Sequence analysis of the Rpt1/p48B gene explained the events leading to lethality in these mutants. The Rpt1/p48B gene, like the majority of the *Drosophila* genes, has a TATA-less promoter (Kutach and Kadonaga 2000). In general, two essential sequences are involved in the transcriptional regulation of genes with a TATA-less promoter: the initiator element (at –2 to +4 bp) and the downstream promoter element (DPE at +28–32 bp). The distance between these elements is critical for the transcriptional regulation of the gene (Kadonaga 2002). The Rpt1/p48B gene contains the consensus DPE element (Fig. 1) and a sequence with homology to the initiator, differing in only two nucleotides. The P-element in the analyzed mutants is inserted just into the DPE element. The P-element insertion induces the duplication of an 8 bp DNA sequence flanking the insertion site. Accordingly, while the original copy of the DPE element is destroyed, one new copy of the DPE element is formed. Following imprecise P-element excision, the critical distance between the hypothetical initiator element and the intact, newly formed DPE is increased by the length of the P-element sequence left behind after the excision (Fig. 1). It was reasonable to suppose that a non-optimum initiator-DPE distance impairs the expression of the Rpt1/p48B gene, leading to pupal or larval lethality, depending on the extent to which the two critical regulatory sequences are displaced. To test this assumption, total RNA was extracted from wild-type and P1 mutant pupae and the concentration of the Rpt1/p48B mRNA was estimated by a semiquantitative RT-PCR analysis. The quantity of the total RNA was normalized for the mRNA content coding the RPL23 ribosomal protein, which was amplified by RT-PCR. Starting with equal amounts of total RNA, Rpt1/p48B cDNA from the wild-type and P1 mutant pupae was amplified by RT-PCR and analyzed on agarose gel. As shown in Fig. 2a, the Rpt1/p48B cDNA prepared from P1 mutant pupae is 32 bp longer than the wild-type cDNA due to the insertion of the P-element sequence. Even with this semi-quantitative test, the concentration of the Rpt1/p48B mRNA seems to be greatly reduced in the mutant pupae. To make this measurement quantitative, the concentrations of the Rpt1/p48B mRNA were measured in the wild-type and P1 mutant pupae by means of an RT-real-time PCR technique. Normalized to 18S rRNA, the concentration of the Rpt1/p48B mRNA was 30-fold lower in the P1 mutant pupae as compared with the wild-type pupae (Fig. 2b). As an internal control, the concentrations of rosy mRNA were also measured in the same RNA preparations under the same conditions. There was no significant difference in the concentration of the rosy mRNA in the wild-type and the P1 mutant pupal RNA. The pupal lethal P1 mutation is, therefore a hypomorphic mutation of the Rpt1/p48B gene. Rpt1/p48B mRNA was undetectable by either semiquantitative or quantitative RT-PCR analysis in the L15 larval lethal mutant (data not shown).

The Rpt1/p48B protein contents of the wild-type and the P1 mutant pupae were estimated by immunoblot analysis. In this experiment, total protein extracts were prepared from wild-type and P1 mutant pupae, and the protein contents of the extracts were normalized to the concentrations of the glycogen phosphorylase household protein estimated by immunoblot staining. Equal amounts of wild-type and P1 pupal protein were loaded for SDS-PAGE and immunoblotted with an anti-Rpt1/p48B antibody. The Rpt1/p48B protein content of the P1 mutant was much less than that of the wild-type pupae (Fig. 3). The polyclonal anti-Rpt1/p48B antibody recognized a closely-spaced triplet both in the wild-type and in the P1 mutant pupal protein extracts. While the electrophoretic mobilities of the individual bands of the triplet were indistinguishable in the wild-type and the

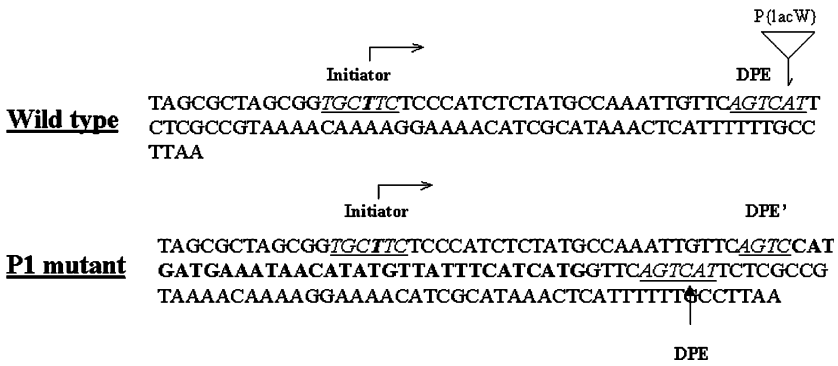

Fig. 1 The DNA sequence of the 5′-end of the Rpt1/p48B gene in wild-type and P1 mutant *Drosophila* stocks. The initiator elements are given in *italics*. The site of P-element insertion is marked by an *arrow* in the wild-type sequence. The P-element sequence left behind in the P1 mutant stock, because of the imprecise P-element excision, is denoted in *bold letters*. The disrupted DPE element and the newly formed DPE element in the P1 mutant are indicated as *DPE′* and *DPE*, respectively

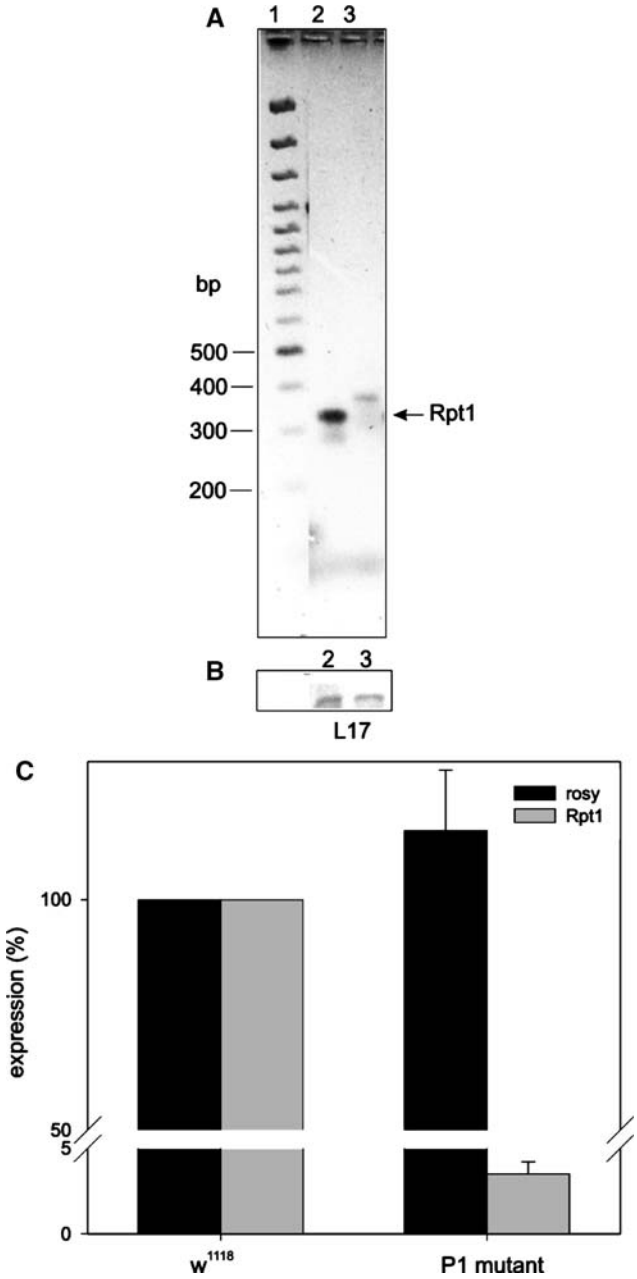

Fig. 2 RT-PCR analysis of the Rpt1/p48B mRNA in wild-type and P1 mutant *Drosophila* pupae. **a** Semiquantitative RT-PCR analysis of the Rpt1/p48B mRNA on 1.5% agarose gel. *Lane 1*: 100 bp marker ladder; *lane 2*: wild-type RT-PCR product; *lane 3*: P1 mutant RT-PCR product. The insert is a loading control showing the RT-PCR product of the RPL23 ribosomal protein mRNA. **b** Loading control of the samples above developed with anti-glycogen phosphorylase antibody. **c** Real-time quantitative RT-PCR analysis of the Rpt1/p48B and the rosy mRNA

mutant pupal protein extracts, the relative intensities of the bands differed significantly. As the human orthologue of the *Drosophila* Rpt1/p48B subunit has been shown to be phosphorylated (Mason et al. 1998), we attempted to prove that the higher molecular weight bands recognized by the polyclonal antibody correspond to different phosphorylated

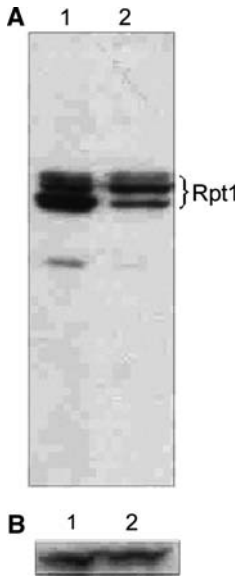

Fig. 3 Immunoblotting analysis of the Rpt1/p48B protein in wild-type and P1 mutant *Drosophila* stocks. **a** Total protein extracts of wild-type (*lane 1*) and P1 (*lane 2*) pupae were separated on 9% SDS-polyacrylamide gel, and blotted proteins were analyzed with anti-Rpt1/p48B polyclonal antibody. **b** Loading control of the samples above developed with anti-glycogen phosphorylase antibody

forms of the protein. For this purpose, total protein extracts of wild-type and P1 mutant pupae were separated by 2D IEF-SDS-PAGE and analyzed by immunoblotting with the polyclonal anti-Rpt1/p48B antibody. As depicted in Fig. 4a, the polyclonal antibody recognizes at least six distinct spots which differ in isoelectric point, strongly suggesting that the Rpt1/p48B protein is multiphosphorylated in the pupae. The 2D gel electrophoresis results confirmed the observation obtained from 1D SDS-PAGE that the relative proportion of the multiple-phosphorylated forms is higher in the mutant pupae as compared with the wild-type. To determine the developmental profile of the multiphosphorylation pattern of Rpt1/p48B protein, total protein extracts of wild-type embryos, pupae and flies were analyzed by 2D IEF-SDS-PAGE. The phosphorylation pattern of Rpt1/p48B protein in pupae and flies was indistinguishable (data not shown), in embryos, however, only mono-, di-, and tri-phosphorylated Rpt1/p48B forms were present in significant quantities, the most highly phosphorylated forms present in pupae and flies were not detectable (Fig. 4b).

By dephosphorylation experiments using potato acid phosphatase we attempted to prove that the different isoforms of Rpt1/p48B separated by 2D IEF-SDS-PAGE correspond to different phosphorylated forms of the subunit. Potato acid phosphatase was used to dephosphorylate two ATPase subunits of the human RP (Mason et al. 1996). To overcome the potential steric hydrance of the ATPase ring by the CP the 26S proteasomes were disassembled into free RP and CP by ATP removal. For this purpose a total embryonic protein extract was fractionated on a Sephadex G25 spin column equilibrated with the reaction buffer of the acid phosphatase. Proteins which appeared in the exclusion volume were incubated with or without 1 U/ml potato acid phosphatase for 1 h at 30°C, fractionated by 2D IEF-SDS-PAGE and analyzed by immunoblotting.

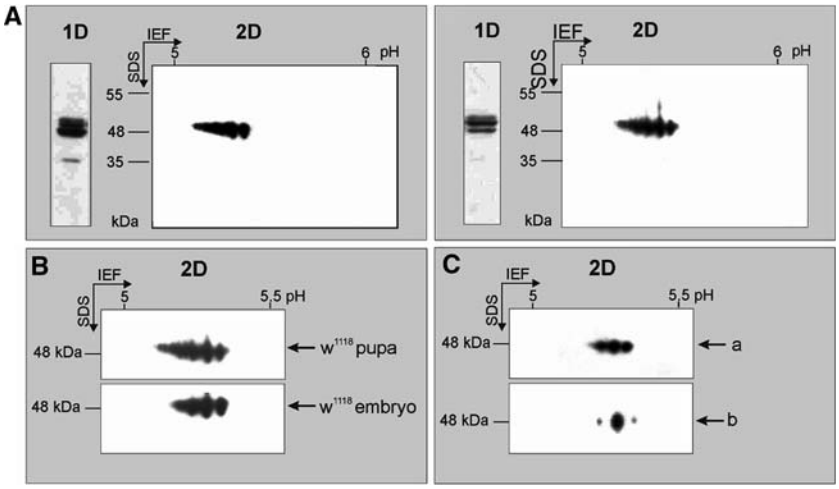

Fig. 4 2D-IEF-SDS-PAGE analysis of the Rpt1/p48B protein. **a** The protein samples shown in Fig. 3 were fractionated on 13 cm long Immobiline DryStrip gels (pH 4–7) in the first dimension, and on 7% SDS-polyacrylamide gel in the second dimension, and immunoblotted with the anti-Rpt1/p48B polyclonal antibody. **b** Total protein extracts prepared from w^{1118} embryos and pupae were fractionated on 2D-IEF-SDS-PAGE and immunoblotted with the anti-Rpt1/p48B polyclonal antibody. **c** Total protein extract prepared from w^{1118} embryos were incubated with or without 1 U/ml potato acid phosphatase, fractionated on 2D-IEF-SDS-PAGE and immunoblotted with the anti-Rpt1/p48B polyclonal antibody. The *upper panel* (marked by *arrow* "*a*") is the control extract, the lower panel (marked by *arrow* "*b*") is the phosphatase treated extract

Following dephosphorylation two Rpt1/p48B isoforms almost completely disappeared and only a single dominant spot was detectable with the antibody (Fig. 4c).

Immunoblotting analysis of total protein extracts prepared from wild-type and P1 mutant pupae with monoclonal antibodies specific for lid or other base subunits of the RP revealed substantial increase in the relative concentrations of two of the tested three RP subunits in the mutant pupae (Fig. 5). Compared with the intensities of the loading control bands (Fig. 5b) there is no change in the concentration of subunit Rpn10/p54.

The Rpt1/p48B subunit is a member of the heterohexameric ATPase ring of the RP which provides a binding surface for the CP during the assembly of the 26S proteasome. Native polyacrylamide gel electrophoresis was used to test how the severe decrease in the Rpt1/p48B protein affects the structure of the 26S proteasome. Total protein extracts prepared from wild-type and P1 mutant pupae were fractionated on 3.8% native polyacrylamide gel, blotted on to a PVDF membrane and developed with different RP- or CP-specific monoclonal antibodies. In the wild-type pupal extracts, the characteristic 26S proteasome pattern was detected (Kurucz et al. 2002), the doubly-capped and the singly-capped 26S proteasome bands reacted with base-, lid- and CP-specific antibodies, and only a small quantity of free CP was present, indicating that the majority of the 20S proteasome particles were assembled into 26S forms (Fig. 6, lanes 1–3). A completely different pattern was observed in the mutant pupal protein extract (Fig. 6, lanes 4–6). Here, the characteristic doublet of the 26S proteasome was completely missing; a strong smeary band reacting with base-, lid- and CP-specific antibodies was present, while the majority of the CP appeared as free particles. This pattern clearly indicates severe damage to the 26S proteasome assembly. The limited concentration of the Rpt1/p48B protein present in the mutant is sufficient only for the

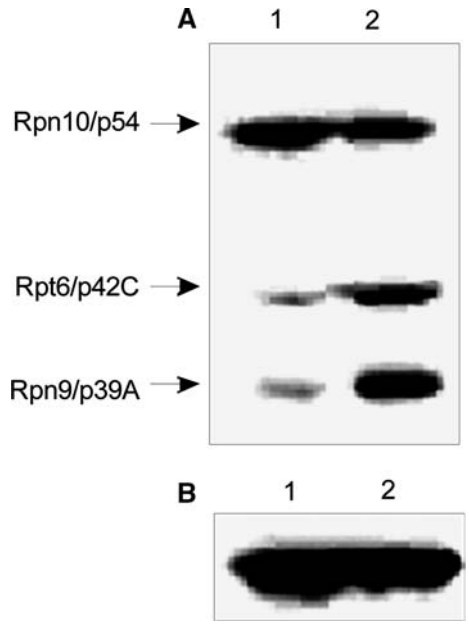

Fig. 5 Up-regulation of RC subunit expression in the P1 mutant Drosophila. **a** Total protein extracts of wild-type (*lane 1*) and P1 mutant (*lane 2*) pupae were fractionated on 8% SDS-PAGE and immunoblotted with a mixture of three monoclonal antibodies specific for the RC subunits Rpn10/p54, Rpt6/p42C and Rpn9/p39A. **b** Loading control of the samples above developed with anti-glycogen phosphorylase antibody

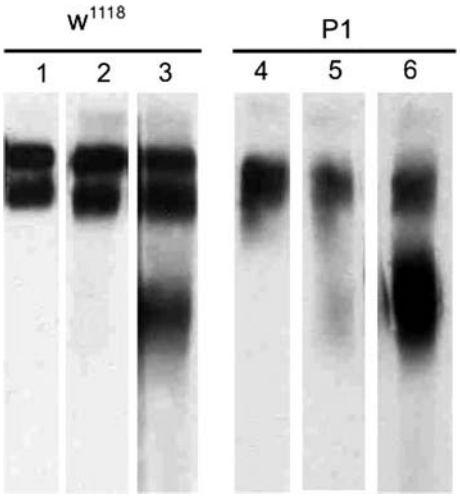

Fig. 6 Native polyacrylamide gel analysis of the structure of the 26S proteasome. Total protein extracts of wild-type (*lanes 1–3*) and P1 mutant (*lanes 4–6*) pupae were fractionated on 3.8% native polyacrylamide gel and immunoblotted with RP base-, RP lid- and CP-specific monoclonal antibodies. *Lanes 1* and *4*: anti-Rpt6/p42C antibody specific for an ATPase subunit; *lanes 2* and *5*: anti-Rpn9/p39A antibody specific for a lid non-ATPase subunit; *lanes 3* and *6*: anti-α1 antibody specific for a CP subunit

Fig. 7 Accumulation of multiubiquitinated proteins in the P1 mutant. **a** Total protein extracts of wild-type (*lane 1*) and P1 mutant (*lane 2*) pupae were fractionated on 7% SDS-PAGE and immunoblotted with anti-ubiquitin polyclonal antibody. **b** Loading control of the samples above developed with anti-glycogen phosphorylase antibody

assembly of a small amount of the intact ATPase ring, and thus the probability of detecting intact 26S proteasomes is negligible. Although a small amount of 20S proteasome is able to bind to the incomplete RPs assembled without the Rpt1/p48B subunit, the 26S proteasome particles formed in this way will have an irregular shape, resulting in the formation of a large mass of smeary band, which reacts with base-, lid- and CP-specific antibodies alike. As a consequence of the reduced concentration of intact 26S proteasome particles, an accumulation of highly multiubiquitinated proteins can be detected in the P1 mutant pupae (Fig. 7a).

Regulatory particle subunits, and especially those belonging to the base subcomplex, are thought to have essential functions inside the chromatin in the regulation of transcription (Ferdous et al. 2001; Gonzalez et al. 2002; Deayoup et al. 2005). By immunofluorescent staining, we analyzed the distribution of the Rpt1/p48B protein in the polythene chromosomes of the salivary gland of wild-type and P1 mutant larvae (Fig. 8). In the wild-type larvae, strong Rpt1/p48B immunostaining was detected over the polythene chromosomes with a characteristic banding pattern (Fig. 8c). Double immunostaining with an anti-RNA polymerase II specific monoclonal antibody revealed that the staining patterns and staining intensities obtained with these antibodies were very similar over the majority of the bands. Inside puffed regions of the polytene chromosomes, however, completely different picture appeared. In consequence of the strong decondensation of the chromatin, the RNA polymerase II staining produced only a faint, narrow band, which is in sharp contrast with the huge accumulation of the Rpt1/p48B protein over the whole area of the puffed regions (arrows in Fig. 8d). The faint, nonspecific staining with the anti-Rpt1/p48B antibody in the P1 mutant larvae is in sharp contrast with the wild-type staining pattern (Fig. 8g). The greatly reduced Rpt1/p48B protein concentration in the mutant resulted in an almost complete depletion of this ATPase protein from the chromatin. The lack of the Rpt1/p48B protein in the polythene chromosomes did not influence the distribution of the RNA polymerase II (Fig. 8f).

The activity of the 26S proteasome is essential for normal cell cycle progression. To determine the role or the contribution of subunit Rpt1/p48B to the overall function of the proteasome in the cell cycle, we analyzed the morphology of mitotic cells in neuroblast preparations from P1 mutant larvae. It was quite surprising that, in contrast with the severe shortage of the functional Rpt1/p48B protein, mitotic defects did not develop in the P1 mutant. In *Saccharomyces cerevisiae* the mutation of the Rpt1 gene results in serious mitotic block and the accumulation of the mitotic cyclins (Ghislain et al. 1993). In agreement with the lack of mitotic defects, the steady state concentration of cyclin A and cyclin B proteins in the P1 mutant and wild type pupae was comparable (Fig. 9).

Discussion

Most of our knowledge on the roles of the individual ATPase subunits of the RP in the proteolytic cycle of the 26S proteasome is derived from *Saccharomyces cerevisiae*. Equivalent mutations of a conserved lysine residue present

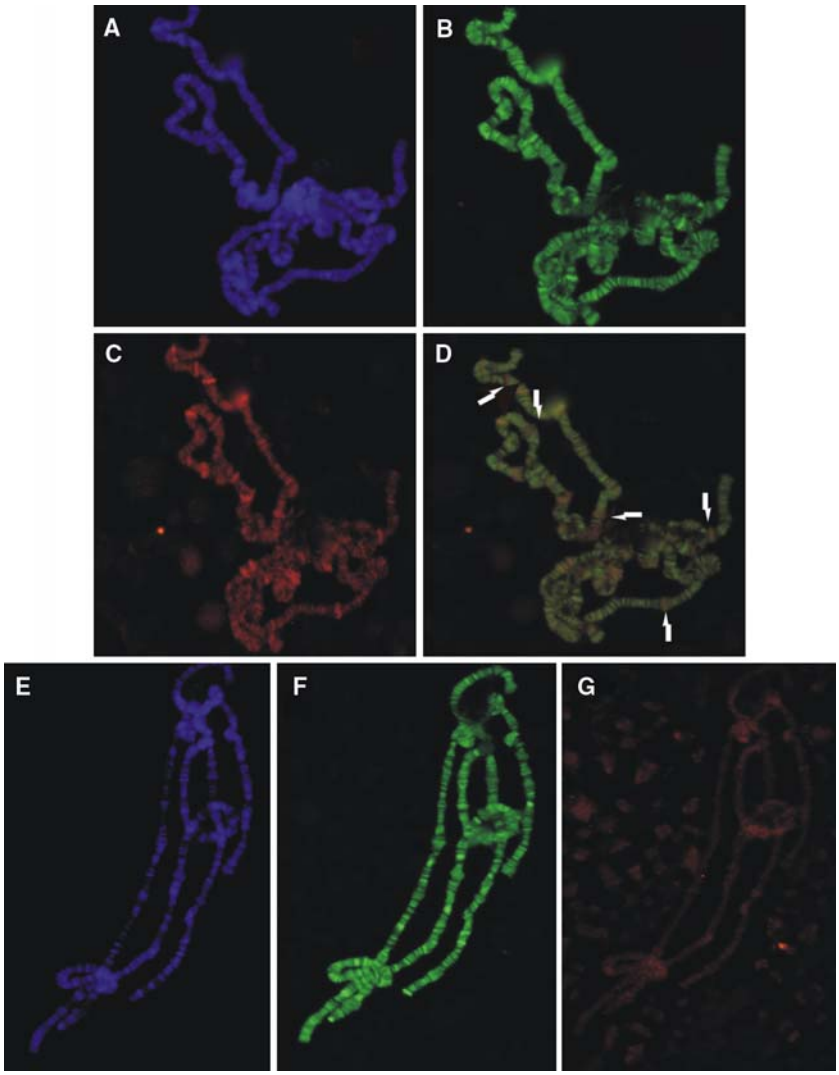

Fig. 8 Immunostaining of salivary gland polythene chromosomes with anti-Rpt1/p48B and anti-RNA polymerase II antibodies. Polythene chromosome spreads of wild-type larval salivary glands were stained with DAPI (**a**) and immunostained with a mixture of anti-Rpt1/p48B polyclonal and anti-RNA polymerase II monoclonal antibodies. The slide was developed with a mixture of FITC-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-rabbit IgG. The localization of RNA polymerase II and Rpt1/p48B is shown in **b** and **c**, respectively. **d** A merge of **b** and **c**. *Arrows* indicate puffed regions with strong Rpt1/p48B accumulation. Polythene chromosome spreads of the P1 mutant larval salivary glands were stained with DAPI (**e**), and immunostained with a mixture of anti-Rpt1/p48B polyclonal and anti-RNA polymerase II monoclonal antibodies. The slide was developed with a mixture of FITC-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-rabbit IgG. The localization of RNA polymerase II and Rpt1/p48B is shown in **f** and **g**, respectively

in the ATP-binding motif of the individual ATPase subunits resulted in distinctly different phenotypes, indicating that the individual ATPase subunits have non-complementary functions (Rubin et al. 1998). This observation was unexpected in view of the high level of sequence homology of the ATPase subunits. Depending on the severity of the phenotype, a hierarchy of phenotypic strength was established. Rpt2 was the most sensitive for the mutation: in this subunit, even a conservative substitution of the lysine residue was lethal, while Rpt1 was the most tolerant as regards the mutation, neither the viability of the yeast cells nor the general proteolytic potency of the 26S proteasome being damaged by even a non-conservative substitution of the critical lysine residue. In view of these observations the lethal phenotype of the hypomorphic Rpt1/p48B mutation is unexpected, and indicates that the structural role of this subunit is just as important as its involvement in the catalytic cycle.

The Rpt1/p48B gene is located inside a long intron of an annotated, but uncharacterized *Drosophila* gene (CG17985). Thus, the phenotype of the original P-element insertions may arise as the combined damage to two independent genes. To analyze the phenotype of the loss of Rpt1/p48B function independently, the P-element of the

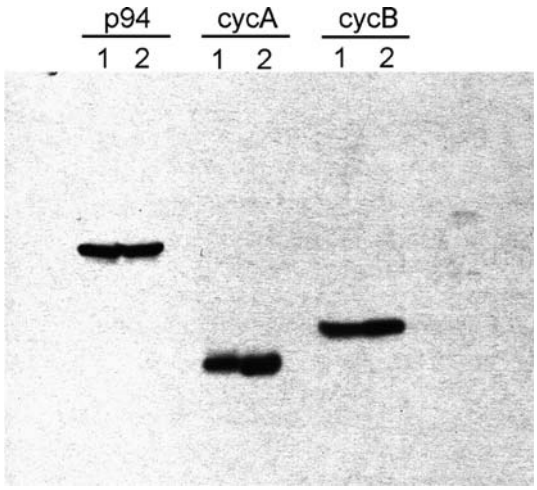

Fig. 9 Immunoblotting analysis of the mitotic cyclin proteins in wild-type and P1 mutant *Drosophila* stocks. Total protein extracts of wild-type (*lane 1*) and P1 mutant (*lane 2*) pupae were fractionated on 8% SDS-PAGE and immunoblotted with anti-glycogen phosphorylase antibody (labeled as *p94*), anti-cyclin A (labeled as *cyc A*) and anti-cyclin B (labeled as *cyc B*) polyclonal antibodies. Immunoblotting with anti-glycogen phosphorylase antibody served as loading control

$Rpt1^{k1110}$ stock was mobilized, and imprecise P-element excisions were selected which affect only the Rpt1/p48B gene, do not harm the annotated CG17985 gene and do not produce second-site mutations elsewhere in the genome. The pupal lethal P1 mutation, which was analyzed in detail, fulfills all these criteria, because a single copy of transgenic Rpt1/p48B gene driven by a ubiquitous promoter could completely rescue its lethal phenotype. The 32 bp long P-element sequence left behind as a result of imprecise P-element excision seriously affected the expression of the Rpt1/p48B gene by displacing the essential DPE regulatory sequence of the gene. The pupal Rpt1/p48B mRNA content of the P1 mutant stock was 30-fold lower than that of the wild-type pupae; hence, P1 is a hypomorphic mutation of the Rpt1/p48B gene. The developmental profile of the cellular concentration of the 26S proteasomes explains the pupal lethal phenotype of mutation P1. In the wild-type *Drosophila* embryos, there is a huge excess of maternally deposited 26S proteasomes, which is gradually depleted during the larval developmental phases. In the third instar larva, the concentration of the 26S proteasomes is very low. During the first few hours of pupal development, the concentration of the 26S proteasomes increases sharply (Szlanka et al. 2003), the newly synthesized 26S proteasomes are probably required for the degradation of the larval proteins. The lack of a sufficient quantity of functional 26S proteasomes, due to the impaired expression of the Rpt1/p48B gene, results in pupal lethality. Although no Rpt1/p48B mRNA can be detected in the L15, L25 and L45 mutant stocks, it cannot be stated that the phenotype of the Rpt1/p48B null mutation is larval lethality in the second instar larval phase, because the Rpt1/p48B transgene could not rescue the homozygous L15 mutation. The failure to rescue these mutations may be a consequence of damage to the CG17985 gene, due to the much longer P-element sequence left behind following the imprecise P-element excision. Essential regulatory elements have been identified in intron regions of *Drosophila* genes. Deletion or rearrangement of these regulatory sequences can substantially influence the expression of these genes (Marais et al. 2005).

The difference in the phenotypic consequences of the Rpt1 mutation in yeast and *Drosophila* is similar to that we have found previously for the deletion of the ubiquitin receptor subunit of the RP. In yeast, the deletion of subunit Rpn10 does not impair the viability and induces only modest changes in the intracellular protein degradation (Van Nocker et al. 1996). Deletion of the orthologue RP subunit in *Drosophila* (Rpn10/p54) results in pupal lethality, serious mitotic defects and a severe disturbance of the intracellular proteolysis (Szlanka et al. 2003).

Native gel electrophoretic analysis of the 26S proteasomes revealed that the assembly of the 26S proteasome in mutant P1 is severely compromised. The majority of the CP was present as free particles in this mutant, indicating that the binding of the CP to the base of the RP is severely impaired. The presence on the native gel of a large smeary immunoreactive mass which reacted with ATPase-, lid- and CP-specific antibodies indicates that, without an intact ATPase ring no well-structured 26S proteasome particle can be assembled. The failure of the assembly is most probably due to the highly reduced Rpt1/p48B protein concentration, although the disturbance of the phosphorylation of the subunit may also contribute to the changes.

Coordinated regulation of the proteasomal gene expression is known to ensure the optimal intracellular concentration of the 26S proteasomes. In the yeast, this regulation is ensured by the RPN4 transcription factor, which is degraded by the 26S proteasome as soon as the proteasome concentration exceeds a critical level (Xie and Varshavsky 2001). Although our knowledge on the molecular details of this regulation is limited, similar feedback regulation should operate in higher eukaryotes: deletion of the Rpn10/p54 subunit of the *Drosophila* RP resulted in a huge overexpression of all the proteasomal subunits (Szlanka et al. 2003), RNA interference-induced down-regulation of subunit Rpn10/p54 resulted in the upregulation of the genes encoding proteasomal subunits (Lundgren et al. 2005). The reduced intracellular concentration of the Rpt1/p48B protein in the P1 hypomorphic mutation also induced a compensatory overexpression of at least two of the tested proteasomal subunits. The overexpression of the RC subunits in the P1 mutants is not as extensive as in the ΔRpn10/p54 mutants probably because the P1 mutant dies earlier during the pupal phase than the ΔRpn10/p54 mutant.

The pupal lethal phenotype of the hypomorphic P1 mutation may be a consequence of a combination of insufficient intracellular proteolysis and impairment of gene expression due to depletion of the Rpt1/p48B protein from the chromatin. In yeast, the degradation of cotranslationally damaged proteins requires the interaction of Rpt1 and the translation elongation factor eEF1 (Chuang et al. 2005). The drop of cellular Rpt1/p48B in our mutants may seriously affect either this salvage process, or the selective degradation of certain short-lived regulatory proteins. In this mutation, however, not all the known substrates of the UPS are affected. Mitotic cyclins do not accumulate in free or multiubiquitinated forms in this mutant. This observation suggests that the individual ATPase subunits of the RP have non-complementary functions essential for the viability of the animal.

The role of the RP and especially its ATPase subunits in the regulation of transcriptional initiation and elongation is well documented (Ferdous et al. 2001; Gonzalez et al. 2002; Deayoup et al. 2005). In this respect the pronounced accumulation of the Rpt1/p48B protein within the puffs of the polytene chromosomes is an important new observation, which supports the notion that proteasomal RP subunits have essential function(s) in the gene expression process. The chromosomal depletion of the Rpt1/p48B protein may damage the transition of the gene expression pattern required for the switch of the larval-pupal developmental phases.

The multiphosphorylation of the *Drosophila* Rpt1/p48B protein is an important new observation. Although the phosphorylation of the ATPase subunits of the human 26S proteasome has been reported previously, the phosphorylation state of the human orthologue of the Rpt1/p48B subunit has not been studied in detail (Mason et al. 1998). The change in relative abundance of the different phosphorylated forms in the P1 mutant, and the change of the phosphorylation state of this subunit during the development suggest that the different phosphorylated species may be involved in distinct functions of the RP.

Our results demonstrate that the Rpt1/p48B subunit of the RP is an essential protein; the decrease in its intracellular concentration due to a hypomorphic mutation results in lethality at pupariation, when increased proteasomal activity is required for the developmental transition.

Acknowledgment This work was supported by the National Scientific Research Fund (OTKA T046177).

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