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A Genetic Suppressor of Two Dominant Temperature-Sensitive Lethal Proteasome Mutants of *Drosophila melanogaster* Is Itself a Mutated Proteasome Subunit Gene

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ABSTRACT

Two dominant temperature-sensitive (DTS) lethal mutants of *Drosophila melanogaster* are *Pros26[1]* and *Pros*β*2[1]*, previously known as *DTS5* and *DTS7*. Heterozygotes for either mutant die as pupae when raised at 29°, but are normally viable and fertile at 25°. Previous studies have identified these as missense mutations in the genes encoding the β6 and β2 subunits of the 20S proteasome, respectively. In an effort to isolate additional proteasome-related mutants a screen for dominant suppressors of *Pros26[1]* was carried out, resulting in the identification of *Pros25[SuDTS]* [originally called *Su(DTS)*], a missense mutation in the gene encoding the 20S proteasome α2 subunit. *Pros25[SuDTS]* acts in a dominant manner to rescue both *Pros26[1]* and *Pros*β*2[1]* from their DTS lethal phenotypes. Using an *in vivo* protein degradation assay it was shown that this suppression occurs by counteracting the dominant-negative effect of the DTS mutant on proteasome activity. *Pros25[SuDTS]* is a recessive polyphasic lethal at ambient temperatures. The effects of these mutants on larval neuroblast mitosis were also examined. While *Pros*β*2[1]* shows a modest increase in the number of defective mitotic figures, there were no defects seen with the other two mutants, other than slightly reduced mitotic indexes.

In eukaryotes most regulated protein degradation is carried out via the ubiquitin–proteasome pathway (Glickman and Ciechanover 2002). In this process, proteins are targeted for destruction by the covalent attachment of a multiubiquitin chain, whereupon they become substrates for a large proteolytic machine called the proteasome. The core of this 26S holoenzyme is a hollow barrel-shaped 20S particle made up of four stacked rings. The two inner rings are each composed of seven distinct β-type subunits (β1–β7) while the two outer rings are each made up of seven different α-type subunits (α1–α7). At each end of the 20S core is a 19S regulatory complex that acts as a gatekeeper, capturing, deubiquinylating, and unfolding tagged substrates and ushering them into the inner chamber of the 20S core where they are hydrolyzed into short peptides.

By controlling the rapid and irreversible turnover of key regulatory proteins, the ubiquitin–proteasome pathway plays important roles in a variety of biological processes, including cell cycle progression (Reed 2003), transcriptional regulation and chromatin remodeling (Kinyamu *et al.* 2005; Hegde and Upadhya 2006), memory and synaptic plasticity (DiAntonio and Hicke 2004), circadian rhythms (Naidoo *et al.* 1999), signal transduction (Ye and Fortini 2000), metabolic regulation (Hampton and Bhakta 1997), antigen processing (Kloetzel 2004), and programmed cell death (Friedman and Xue 2004). This pathway also carries out an important "housekeeping" function, by ridding cells of potentially harmful abnormal proteins that arise as the result of mutation, misfolding, or postsynthetic damage (Kostova and Wolf 2003).

One way to investigate the biological roles of the ubiquitin–proteasome pathway is to use a mutational approach to disrupt proteasome function and to then assess the effects on the process of interest. Since this pathway is critically involved in so many cellular events most proteasome null mutants are lethals. Thus, the most useful alleles for manipulating proteasome function are hypomorphic (leaky) or conditional mutants. In *Drosophila melanogaster*, two such mutants are *Pros26[1]* and *Pros*β*2[1]*. These were isolated in a screen for dominant temperature-sensitive (DTS) lethal mutants and originally named *DTS5* and *DTS7* (Holden and Suzuki 1973). Subsequent study revealed that each mutation results in a single-amino-acid substitution in a 20S proteasome subunit (the β6 and β2 subunits, respectively) (Saville and Belote 1993; Smyth and Belote 1999). The phenotypes of both mutants are similar, with heterozygotes raised at 29° dying during the pupal stage with numerous defects including reduced abdominal

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histoblast proliferation and a failure of head eversion. At 25°, such flies develop normally and are fully viable and fertile. These two mutants exhibit strong synthetic lethality in that double heterozygotes die as early stage larvae at ≥22° (Smyth and Belote 1999). Genetic and biochemical evidence suggests that these mutants act in a dominant-negative manner to interfere with proteasome function (Saville and Belote 1993; Covi *et al.* 1999; Schweisguth 1999; Smyth and Belote 1999; Speese *et al.* 2003; K. Vitale and J. Belote, unpublished data). Null alleles of these loci are recessive, non-temperature-sensitive, early larval lethals (Smyth and Belote 1999).

The dominant temperature-sensitive nature of these mutants makes them useful for manipulating proteasome function *in vivo* (*e.g.*, see Huang *et al.* 1995; Henchoz *et al.* 1996; Heriche *et al.* 2003). For example, by shifting the culture temperature of heterozygotes during development it is possible to disrupt proteasome function in a stage-specific manner. Because *Pros26*^{1} and *Pros*β*2*^{1} act in a dominant-negative manner, it is also possible to target their effects to particular cells or tissues using the UAS/GAL4 binary system of Brand and Perrimon (1993). A number of *UAS-Pros26*^{1} and *UAS-Pros*β*2*^{1} transgenic lines have been generated and used for this purpose (Schweisguth 1999; Belote and Fortier 2002; Chan *et al.* 2002; Khush *et al.* 2002; Speese *et al.* 2003; Shulman and Feany 2003; Tang *et al.* 2005). These transgenic lines provide a complementary approach to the use of proteasome inhibitors (Myung *et al.* 2001) to investigate the roles that proteasome-related proteolysis plays during development. This genetic approach has some advantages over the use of exogenous inhibitors, whose specific delivery only to the cells of interest is difficult.

In an effort to isolate additional useful proteasome mutants, a screen for dominant suppressors of the *Pros26*^{1} mutant was carried out. It was hypothesized, on the basis of classic studies of suppression in *Saccharomyces cerevisiae* (*e.g.*, see Huffaker *et al.* 1987; Adams and Botstein 1989; Novick *et al.* 1989), that dominant extragenic suppressors of this conditional mutant, encoding a component of a multisubunit complex, might represent useful hypomorphic, conditional, or gain-of-function alleles of other proteasome components. Here we describe the isolation and genetic characterization of a dominant suppressor of both *Pros26*^{1} and *Pros*β*2*^{1} and show that it is a mutant allele of *Pros25*, encoding the α2 subunit of the 20S proteasome.

MATERIALS AND METHODS

Fly culture: *D. melanogaster* strains were cultured on standard media containing cornmeal, dextrose, sucrose, yeast, and agar. Stocks were obtained from the Bloomington Stock Center, and their descriptions are available on the FlyBase server (http://flybase.bio.indiana.edu/). *P*-element transformation was done using standard procedures with w^{1118} as the host strain.

Isolation and mapping of the *Su(DTS)* mutant: *Pros26*^{1}*/TM3, Sb p*^{p} *e* males were fed for 24 hr on a solution containing 0.2 mg/ml of l-ethyl-1-nitrosourea (ENU; Sigma, St. Louis) in 2% sucrose. Following a 24-hr recovery period on standard, yeasted Drosophila medium, the males were mated to *Pros26*^{1}*/TM3, Sb p*^{p} *e* virgin females (~25 pairs/bottle). After a day at room temperature, bottles were placed at 29° and parents removed at day 7. Rare survivors were mated to *Pros26*^{1}*/TM3, Sb p*^{p} *e* mates and progeny reared at 29° to confirm that the original survivor was not an "escaper." Only lines that gave robust survival at 29° were kept for analysis. The *Su(DTS)* mutant was separated from the *Pros26*^{1} allele by meiotic recombination and mapped using the multiply marked *ru h th st cu sr e*^{s} *ca* chromosome. A second meiotic recombination mapping experiment used the *st ri p*^{p} chromosome. For the two mapping experiments, the third chromosome carrying the *Su(DTS)* mutant was made heterozygous over the multiply marked chromosome in females, which were then crossed to *ru h th st cu sr e*^{s} *Pri ca/TM6B, Bri*^{1} or *st ri Ki p*^{p} males, respectively. Various recombinant males were then selected and tested for the ability to suppress the dominant temperature-sensitive lethality of *Pros26*^{1} by mating them to *Pros26*^{1} *pb p*^{p}*/TM3, Sb p*^{p} *e* virgins and raising the offspring at 29°. All recombinant chromosomes that carried *Su(DTS)* were homozygous lethal. The recessive lethal was therefore mapped by deficiency mapping using the following: *Df(3R)by62*, *Df(3R)cu*, *Df(3R)M-Kxl*, *Df(3R)T-32*, *Df(3R)ry85*, *Df(3R)MRS*, and *Df(3R)red3l*.

The *Su(DTS)* mutation was mapped more precisely using *P*-element-mediated site-specific male recombination (Chen *et al.* 1998). Females of genotype *y w; CyO, H{P{*Δ*2-3}HoP2.1/Bc*^{1} *Egfr*^{E1}*; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e* were crossed to males of various *P*-element insertion lines to generate *y w; CyO, H{P{*Δ*2-3}HoP2.1/+; st ri p*^{p} *Su(DTS) e ca/P-element* males. These were then individually crossed to *st e* virgin females and offspring were scored for *st*$^{+}$ *e* and *st e*$^{+}$ recombinants. Recombinant males were each tested first for the presence of the recessive lethal associated with *Su(DTS)* by crossing them to *st ri p*^{p} *Su(DTS) e ca/TM3, Sb* females. Once larvae were apparent, the recombinant males were then removed and tested for the presence of the dominant suppressor of DTS by mating them to *Pros26*^{1} *pb p*^{p}*/TM3, Sb p*^{p} *e* and raising the offspring at 29°. The *P* elements used for mapping *Su(DTS)* included: *P{PZ}srp[01549]*, *P{lacW}Vha55[j2E9]*, *P{PZ}svp[07842]*, *P{PZ}l(3)09656[09656]*, *P{PZ}l(3)rM060[rM060]*, *P{PZ}tws[02414]*, and *P{PZ}l(3)10615[10615]*. In all cases, both phenotypes mapped to the same side of the *P* element.

General molecular procedures: All standard molecular techniques were done essentially as described in Sambrook *et al.* (1989). Plasmid purification was done using the Wizard *Plus* Miniprep kit (Promega, Madison, WI). DNA sequencing was performed by the Syracuse University Biology Department Sequencing Facility (Syracuse, NY) or the BioResource Center at Cornell University (Ithaca, NY).

Cloning of the *Pros25* gene: Genomic DNA was extracted from non-Tubby third instar larvae from a cross of *st ri p*^{p} *Su(DTS) e*^{s} *ca/TM6B, Tb e ca* males and females according to the method of Gloor and Engels (1992). *Pros25* sequences were PCR amplified using the following primers: PROS25-5′-1, ATCAAATCACTGCATTTGCGG, and PROS25-3′-4, CTTAG CTTGTGGTAATCTTAGC, and ligated into the pGEM-T Easy vector (Promega) to give pGEM-T Easy/Pros25^{SuDTS}. Clones from two independent PCR reactions were sequenced using the T7 and M13R primers corresponding to vector sequences and two internal primers, PROS25-5′-2, GAGATGATCTAC AACCACATC, and PROS25-3′-3, GATCAGTAGGGAAACGCC AAA. To clone the *Pros25* allele present on the unmutagenized

chromosome, genomic DNA was extracted from non-Tubby larvae raised at 18° from a cross of *Pros26^{1}/TM6B, Tb e ca* males and females, and the *Pros25* gene was PCR amplified and cloned as above to give pGEM-T Easy/Pros25^{+}.

***P*-element transformation constructs:** A BAC clone (RPCI-98 28.I.14) containing the *Pros25* genomic region was obtained from Children's Hospital Oakland Research Institute (Hoskins *et al.* 2000) and DNA was prepared using a QIAGEN (Valencia, CA) plasmid midi-prep kit. The published sequence of this clone (accession no. AC007594) predicted a 5.7-kb *Kpn*I–*Sal*I fragment containing the *Pros25* gene region. BAC DNA was therefore treated with these enzymes and the 5.7-kb fragment was gel purified and ligated into pBluescriptKS+ (Stratagene, La Jolla, CA) to obtain pBS/Pros25-5.7KS. A 2.0-kb *Kpn*I–*Bam*HI fragment from this clone was subcloned into pBluescriptKS+ to give pBS/Pros25-2.0KB. The 2.0-kb *Kpn*I–*Bam*HI fragment was then subcloned into the pW8 transformation vector to give pW8/Pros25-2.0KB. The construct was introduced into the genome by *P*-element transformation and several transgenic lines were obtained.

The inserts from pGEM-T Easy/Pros25^{+} and pGEM-T Easy/Pros25^{SuDTS} were cut out with *Eco*RI and cloned into the pUAST vector to give pUAST/Pros25^{+} and pUAST/Pros25^{SuDTS}, and multiple transgenic lines were obtained for each. Strains containing *UAS-Pros26^{1}* or *UAS-Pros*β*2^{1}* transgenes are described in Belote and Fortier (2002). Creation of the *UAS-Pros29* transgenic line is described in Ma (2001).

Cytology: Metaphase figures were prepared according to the protocol of Gatti and Baker (1989). Brains from late third instar larvae raised at 29° were dissected in 0.7% saline, incubated in 0.5×10^{-5} M colchicine in 0.7% NaCl for 1 hr at room temperature, and placed in 0.5 M sodium citrate for 7 min. After fixing in methanol:acetic acid:dH_2O (11:11:2) for 30–45 sec the brains were placed in a 5-μl drop of aceto-orcein stain (2% orcein in 45% acetic acid) on a siliconized coverslip and then squashed on a microscope slide. For observation of anaphase figures and determination of mitotic indexes, the colchicine and sodium citrate steps were omitted. Slides were examined with a Zeiss Axioplan phase contrast microscope using a 100× oil immersion objective. Mitotic indexes (MI) were calculated as the number of mitotic figures per microscope field. At least six slides were scored for each genotype, with the number of fields scanned per slide varying between 40 and 108, depending on the size of the brain squash.

Construction of heat-shock-inducible unstable and stable GFP reporter transgenes: An unstable enhanced green fluorescent protein (EGFP) reporter was made by fusing a portion of the Drosophila Notch protein containing its PEST degradation signal (*i.e.*, the Notch-intracellular, Nintra, domain) to the C terminus of EGFP. First, a fragment of the Drosophila *Notch* gene, encoding the carboxy-terminal 178 amino acids of Notch, was PCR amplified from genomic DNA using primers BNIN, CGGATCCTCGAAGAATAGTG CAATAATGCAAACG, and NINN, GCGGCCGCGATATTCAA CATACCAAATCATCCAGATCA, and ligated into the pGEM-T Easy vector (Promega) to give pGEM-T/NintraBN. The coding region of pEGFP (Clontech, Palo Alto, CA) was PCR amplified using primers BEGFP, GGATCCGAATTCGCCAC CATGGTGAGCAAGGGCGAGGAG, and GFPB, GGATCCTT GTACAGCTCGTCCATGCCGAGAGTGATCCC, and ligated into pGEM-T Easy. The EGFP sequence was then cut out with *Bam*HI and ligated into the *Bam*HI site of pGEM-T/NintraBN to give pGEM-T/EGFP-Nintra. A clone with EGFP inserted in the correct orientation was digested with *Not*I and the 1.4-kb fragment was subcloned into pCaSpeR-hs (Thummel and Pirrotta 1992) to yield pCasper-hs/EGFP-Nintra. This was introduced into the genome by *P*-element-mediated germline transformation and several transgenic lines were established. One line, *w P{hs-EGFP-Nintra,w^{+}}15(X)*, which carries the transposon on the X chromosome, gave good heat-shock-inducible expression of EGFP-Nintra and was used for the experiments described here. As a control, a stable, heat-shock-inducible EGFP construct was made by subcloning the *Hin*cII/*Not*I restriction fragment of pEGFP into the *Hpa*I/*Not*I sites of pCaSpeR-hs to give pCasper-hs/EGFP. One transgenic line, *w; P{hs-EGFP, w^{+}}12(3)* was generated, which gave good heat-shock-inducible expression of EGFP.

***In vivo* monitoring of GFP stability:** To assess the degradation of EGFP-Nintra in the presence of dominant proteasome mutants, females of genotype *w P{hs-EGFP-Nintra}15(X); P{w$^{+\,mW.hs}$=GawB}ptc$^{559.1}$* were crossed at 29° to the following males: *w; P{UAS-Pros*β*2^{1}, w^{+}}2B(3), w: P{UAS-Pros26^{1}, w^{+}}6A(3), w; P{UAS-Pros25^{+}, w^{+}}4A(2), w; P{UAS-Pros25^{SuDTS}, w^{+}}2A(2), w: P{UAS-Pros29, w^{+}}1(2), w; P{UAS-lacZ, w^{+}}(2)*, and *w; P{UAS-Pros25^{SuDTS}, w^{+}}2A(2); P{UAS-Pros*β*2^{1}, w^{+}}2B(3)*. Late third instar larvae were placed in prewarmed small petri dishes containing grape juice/agar and heat-shocked by placing them in a 37° incubator for 30 min. The larvae were then transferred to a 29° dish and allowed to recover for 4 hr. Larvae were dissected in phosphate-buffered saline (PBS, 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) and the carcasses, with wing discs exposed, were fixed in 4% paraformaldehyde (EM Sciences) in PBST (PBS with 0.5% Triton X-100) for 1 hr. After washing 3 × 20 min in PBST, the larvae were treated for 1 hr at 37° with RNase A (0.5 mg/ml in PBST) and then washed 3 × 20 min with PBST. They were then transferred to PBS containing 1 μg/ml TOPRO-3 DNA stain (Invitrogen, San Diego) for 30 min and washed briefly in PBS. The wing discs were then dissected and mounted in ProLong anti-fade mountant (Invitrogen). A Zeiss LSM5 Pascal confocal microscope was used for fluorescence imaging.

Structural analysis of the 20S proteasome: Mutations were mapped onto the structure of the bovine 20S proteasome, Protein database code 1IRU (Unno *et al.* 2002), using the PyMOL molecular graphics system (http://www.pymol.org).

RESULTS

Isolation of a dominant suppressor of *Pros26^{1}*: The *Pros26^{1}* mutant is a highly penetrant DTS lethal that also acts as a recessive lethal at all temperatures (Holden and Suzuki 1973; Saville and Belote 1993). When heterozygotes are reared at 29°, very few, if any, survive to adulthood. In an effort to identify new genes that play roles in the ubiquitin–proteasome pathway, a screen for dominant suppressors of the *Pros26^{1}* DTS lethality was carried out (Figure 1). In this experiment, *Pros26^{1}/TM3, Sb p^{p} e* males were fed ENU and mated to *Pros26^{1}/TM3, Sb p^{p} e* females. The F_1 were raised at 29°. In the absence of any new mutation, no survivors were expected. To estimate the number of F_1 individuals being screened, control crosses were maintained at 25° and scored for survival of *Pros26^{1}/TM3, Sb p^{p} e* offspring. Of ~4000 *Pros26^{1}/TM3, Sb p^{p} e* F_1 individuals raised at 29°, ~10 adult flies were recovered. Most of these were sick and sterile and could represent rare escapers that somehow avoided the usual lethality. The few fertile F_1 adults were crossed to *Pros26^{1}/TM3, Sb p^{p} e* mates and progeny were raised at 29° to see if the suppression of DTS lethality was heritable and reproducible. Three balanced stocks were established for further analysis.

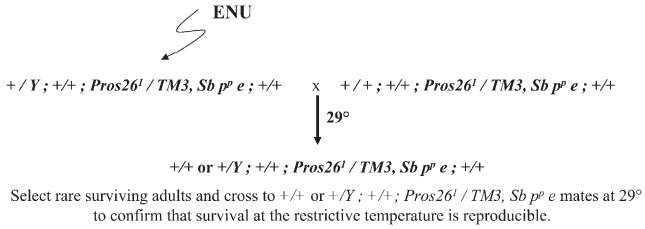

FIGURE 1.—Crossing scheme for the mutant screen for suppressors of *Pros26¹* DTS lethality.

Although the design of this screen could have resulted in the isolation of suppressors of DTS on any of the chromosomes, all three mutants mapped to chromosome 3.

Of the three DTS suppressor mutants, two were "pseudorevertants" that had picked up a loss-of-function mutation of *Pros26*. These were characterized by early larval lethality at 25° when the mutant chromosome was placed over either *Pros26¹* or *Df(3L)st-j7* (a deletion of the *Pros26* gene region) and by the lack of any synthetic lethal interaction with *Prosβ2¹*. These suppressor mutants also could not be separated by recombination from the *Pros26¹* gene present on the original mutagenized chromosome. One of these, *Pros26^rv10e*, was further characterized molecularly by PCR amplifying and sequencing the *Pros26* gene and, as expected, it was found to carry a newly induced mutation in the *Pros26* coding region that presumably results in a null allele; *i.e.*, it was a nonsense mutation at codon position 78. Such pseudorevertants are expected products of this screen since a newly induced null mutation in the original *Pros26¹* allele would no longer act in a dominant-negative fashion.

One mutant line had properties suggesting that it carried a third-chromosome second-site dominant suppressor of *Pros26¹*. Flies heterozygous for this mutagenized chromosome carrying *Pros26¹* were reproducibly viable when reared at the normally restrictive temperature of 29°. In addition, when the chromosome carrying this mutant [referred to here as *Su(DTS)*] and *Pros26¹* was placed over either *Pros26¹* or *Df(3L)st-j7* it was weakly viable at 18°, and it still displayed a synthetic lethal interaction with *Prosβ2¹* at 25°. These phenotypes would not be expected for a pseudorevertant loss-of-function *Pros26* allele, suggesting that the original *Pros26¹* allele was still present on this chromosome. Most importantly, the *Su(DTS)* mutation could be separated by recombination from the *Pros26¹* locus, which maps to 3-45 (or salivary gland chromosome region 73B1). Initial mapping experiments showed that the *Su(DTS)* mutant resides between the *scarlet* and *stripe* genes at approximately 3-50. A second meiotic recombination mapping experiment localized *Su(DTS)* close to, but to the right of, *pink*. The interval between *pink* and *stripe* corresponds to cytogenetic region 85A6–90E4. During the course of these experiments, lines were established that carried *Su(DTS)* but no longer carried the *Pros26¹* mutant. In all cases, the recombinant lines were homozygous lethal, suggesting that *Su(DTS)*, in addition to acting as a dominant suppressor of *Pros26¹*, has a recessive lethal phenotype as well (also, see below).

Genetic interactions of the *Su(DTS)* mutant: The *Su(DTS)* mutant is a very effective suppressor of the DTS lethal effect of *Pros26¹*. In the absence of *Su(DTS)*, individuals carrying *Pros26¹* die during the late larval or pupal stages when reared at 29° (Table 1, line A), while such flies are completely rescued if one copy of *Su(DTS)* is present (Table 1, line B). The recessive lethal phenotype of *Pros26¹* is only partially suppressed by *Su(DTS)* and the survivors are slow developing and sick (Table 1, lines C and D). Surprisingly, the *Su(DTS)* mutant also completely rescues the DTS lethal phenotype associated with the other DTS proteasome mutant, *Prosβ2¹* (Table 1, lines E and F). In addition, there is some rescue from the early larval lethality exhibited by + *Pros26¹/Prosβ2¹* + *trans*-heterozygotes, although survivors have small, thin bristles and are infertile (Table 1, lines G and H). The finding that *Su(DTS)* is able to suppress the DTS phenotype of two different proteasome mutants strongly

TABLE 1

Genetic interactions among *Pros26¹*, *Prosβ2¹*, and *Su(DTS)*

Cross	Temperature	Tubby ebony	Tubby	Non-Tubby
A. *w; Pros26¹ pb pᵖ/TM6B, Tb e ca* × *w; +/+*	29°	—	452	0
B. *w; Pros26¹ Su(DTS)/TM6B, Tb e ca* × *w; +/+*	29°	—	316	298
C. *w; Pros26¹ pb pᵖ/TM6B, Tb e ca* × *w; Pros26¹ pb pᵖ/TM6B, Tb e ca*	25°	—	340	0
D. *w; Pros26¹ Su(DTS)/TM6B, Tb e ca* × *w; Pros26¹ pb pᵖ/TM6B, Tb e ca*	25°	—	245	18
E. *w; Prosβ2¹ st tra in pᵖ′/TM6B, Tb e ca* × *w; +/+*	29°	—	429	0
F. *w; Prosβ2¹ st tra in pᵖ′/TM6B, Tb e ca* × *w; st ri pᵖ Su(DTS) e ca/TM6B, Tb e ca*	29°	253	0	305
G. *w; Pros26¹ pb pᵖ/TM6B, Tb e ca* × *w; Prosβ2¹ st tra in pᵖ′/TM6B, Tb e ca*	25°	—	283	0
H. *w; Pros26¹ Su(DTS)/TM6B, Tb e ca* × *w; Prosβ2¹ st tra in pᵖ′/TM6B, Tb e ca*	25°	—	168	14
I. *w; st ri pᵖ Su(DTS) e ca/TM6B, Tb e ca* × *w; st ri pᵖ Su(DTS) e ca/TM6B, Tb e ca*	25°	463	—	0
J. *w; Pros26¹ Su(DTS)/TM6B, Tb e ca* × *w; st ri pᵖ Su(DTS) e ca/TM6B, Tb e ca*	25°	137	159	28
K. *w; Prosβ2¹ Su(DTS)/TM6B, Tb e ca* × *w; st ri pᵖ Su(DTS) e ca/TM6B, Tb e ca*	25°	187	201	0

suggests that its function is closely related to the ubiquitin–proteasome pathway.

Additional crosses were carried out to investigate the genetic interactions among these three mutants. It was found that not only does the *Su(DTS)* mutant suppress the DTS lethality of *Pros26*^{1}, but the *Pros26*^{1} mutant also acts to suppress the recessive lethality of *Su(DTS)* (Table 1, lines I and J). While this suppression effect is weak, it is significant in that in the absence of *Pros26*^{1}, no homozygous *Su(DTS)* flies have ever been seen to survive to adulthood. Unlike *Pros26*^{1}, the *Pros*β*2*^{1} mutant did not appear to suppress the recessive lethality of *Su(DTS)* (Table 1, line K).

Molecular identification of the *Su(DTS)* mutant: As the first step toward its molecular identification, the *Su(DTS)* mutant was precisely mapped using *P*-element-mediated site-specific male recombination (Chen *et al.* 1998). This analysis (described in materials and methods) revealed that both phenotypes of *Su(DTS)*, *i.e.*, the recessive lethality and the dominant suppression of the two DTS proteasome mutants, were caused by mutation(s) in the interval between *P{PZ}svp* at 87B4–5 and *P{lacW}Vha55* at 87C2–3, consistent with the earlier mapping results. Chromosome deficiencies were also used to map the recessive lethal phenotype of *Su(DTS)* to the same region (Figure 2A).

On the basis of the molecular positions of the defining *P*-element transposon insertions, the *Su(DTS)* mutant was delimited to an ~350-kb region containing 63 annotated genes (Drysdale *et al.* 2005). Among the genes in this interval are several that have a recognizable relationship to protein stability and degradation, including four Hsp70 protein chaperone genes (*Hsp70Ba*, *Hsp70Bb*, *Hsp70Bc*, and *Hsp70Bd*), two peptidase genes (*CG10041* and *Dip-C*), and a putative ubiquitin-like protein-activating enzyme (*Aos1*). The most interesting candidate gene in this region was *Pros25*, which encodes the α2 subunit of the 20S proteasome (Seelig *et al.* 1993). Because Pros25 is a component of the same macromolecular complex that contains both Pros26 (β6 subunit) and Prosβ2 (β2 subunit) it seemed likely that *Su(DTS)* was a mutant allele of this gene. To test this, the *Pros25* locus was PCR amplified from homozygous *Su(DTS)* larvae and analyzed by DNA sequencing. This revealed that there was a G-to-A transition mutation resulting in the replacement of a cysteine with a tyrosine at amino acid position 212. This cysteine is highly conserved, being found in every metazoan α2 subunit that has been sequenced, including those from *C. elegans*, *A. gambii*, *X. laevis*, *G. gallus*, and *H. sapiens*. To confirm that this amino acid substitution is not a naturally occurring polymorphism, the *Pros25* gene was amplified and sequenced from the *Pros26*^{1}-bearing chromosome carried in the stock used for the suppressor mutant screen. The results showed that the mutation was not present in the original stock and most likely was generated by the ENU treatment. These results strongly suggest that the

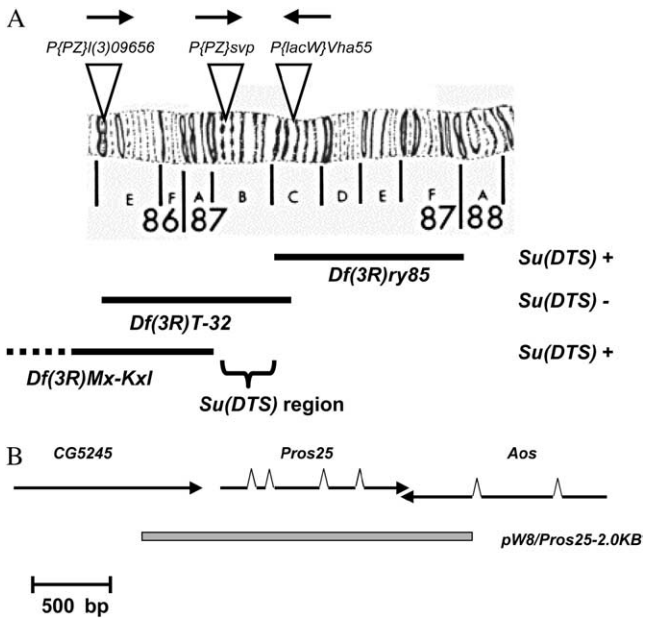

Figure 2.—Cytogenetic mapping and molecular identification of the *Su(DTS)* mutant. (A) The positions of three of the *P*-element insertions used to map *Su(DTS)* by site-specific male recombination are shown. The arrows show which side of the element the *Su(DTS)* mutant was found to map. The solid bars represent the deleted regions of three deficiencies that were used to map the recessive lethal associated with *Su(DTS)*. (B) The molecular structures and orientations of *Pros25* and its flanking genes are shown. The shaded bar represents the subcloned fragment that was used for the transgenic rescue experiments.

Su(DTS) mutant is an allele of *Pros25*. To confirm this, a 2.0-kb *Kpn*I/*Bam*HI restriction fragment containing the wild-type *Pros25* gene was isolated from a recombinant BAC clone and subcloned into the pW8 transformation vector, and transgenic lines were established (Figure 2B). The pW8/Pros25-2.0KB transgene was able to completely rescue transgenic flies from the recessive lethality associated with *Su(DTS)* (Table 2), strongly supporting the idea that the recessive lethal phenotype is the result of mutation in *Pros25*.

To address whether the *Pros25* mutation is also responsible for the dominant suppressor of DTS phenotype, *Pros25*$^{+}$ transgenic flies carrying an endogenous copy of *Su(DTS)* were crossed to *Pros*β*2*^{1} and the offspring were raised at 29°. If the suppressor of DTS phenotype is due to the mutation in *Pros25*, then an extra copy of *Pros25*$^{+}$ should counteract this effect. Indeed, in the presence of a transgenic copy of *Pros25*$^{+}$, a single dose of *Su(DTS)* was unable to suppress the DTS phenotype of *Pros*β*2*^{1} (Table 3, lines B and F). These experiments demonstrate that both phenotypes of *Su(DTS)* are due to the mutation in *Pros25*, and the mutant is therefore named *Pros25*^{SuDTS}. This represents the first mutant allele of this gene that has been described.

Additional phenotypic effects of the *Pros25*^{SuDTS} mutation: To examine the recessive lethal phenotype

TABLE 2

Rescue of the *Su(DTS)* recessive lethal phenotype by a *Pros25*$^{+}$ transgene

F_1: genotype	No.
Cross (at 25°): *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e* × *w; +/+; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e*	
A. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e*	45
B. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/st ri p*^{p} *Su(DTS) e ca*	22
C. *w; +/+; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e*	36
D. *w; +/+; st ri p*^{p} *Su(DTS) e ca/st ri p*^{p} *Su(DTS) e ca*	0

of *Pros25*^{SuDTS} in more detail, eggs were collected from crosses of *w; st ri p*^{p} *Pros25*^{SuDTS} *e ca / TM6B, Tb* parents and hatching frequency and development of non-Tubby larvae and pupae were monitored at 25°. This analysis showed that there was little if any embryonic lethality associated with *Pros25*^{SuDTS} homozygotes, but the lethal period was polyphasic throughout the larval and pupal stages. Some of the homozygous larvae exhibited slow growth rate and sluggish behavior, while others progressed through the larval stages with normal appearance and behavior, although pupation was usually delayed a day or two. As pupae, most mutant individuals failed to develop to the late stages, although a few became pharate adults. None of the homozygotes eclosed. In some cases, the dying homozygous larvae exhibited necrotic gut tissue, similar to what has been described for larvae subjected to lethal heat shocks (Krebs and Feder 1997).

A loss-of-function mutation in another essential proteasome gene, *Pros54* encoding the Rpn10 subunit of the 19S regulatory cap, has been shown to cause strong mitosis-defective phenotypes, as seen in dividing larval neuroblasts (Szlanka *et al.* 2003). To investigate whether the *Pros25*^{SuDTS}, *Pros26*^{1}, and *Pros*β*2*^{1} proteasome mutants also exhibit mitotic defects, brain squashes from dying mutant larvae were prepared and examined for abnormalities (Figure 3). *Pros26*^{1} and *Pros*β*2*^{1} heterozygotes, reared at the restrictive temperature of 29°, were selected as late third instar larvae, and brain squashes were prepared either with colchicine treatment, to examine metaphase figures for evidence of aneuploidy and polyploidy, or without such treatment, to determine the mitotic index and to examine anaphase figures. For *Pros25*^{SuDTS}, brain squashes were prepared from late third instar homozygous larvae raised at 25° or 29°. Unlike what was reported for the *Pros54*$^{\Delta p54}$ mutant (Szlanka *et al.* 2003), there were few mitotic defects associated with these three proteasome mutants. That is, *Pros26*^{1}/+ and *Pros25*^{SuDTS}/*Pros25*^{SuDTS} larval brains showed no apparent instances of polyploidy or aneuploidy, and chromosome morphology and anaphase figures appeared normal (Figure 3, A and B). For *Pros*β*2*^{1}/+ larvae, there was a higher than background incidence of tetraploid and aneuploid metaphase figures (Figure 3, C–E), although most (>95%) mitotic spreads were normal. A few mitotic figures showed precocious chromatid separation (Figure 3F). The mean MI and standard errors obtained for the three mutant genotypes were *Pros25*^{SuDTS}/*Pros25*^{SuDTS} = 0.97 ± 0.17, *Pros26*^{1}/+ = 1.00 ± 0.14, and *Pros*β*2*^{1}/+ = 1.07 ± 0.20. These are somewhat reduced compared to those of wild-type controls (mean MI =1.43 ± 0.18), and this probably reflects the slower developmental rate of the mutant larvae.

A comparison was made between the lethal phenotypes of *Pros25*^{SuDTS}/*Pros25*^{SuDTS} and *Pros25*^{SuDTS}/*Df(3R)T-32* and it was seen that both genotypes showed similar delayed larval development and sluggish behavior, but that *Pros25*^{SuDTS}/*Df(3R)T-32* larvae were more severely affected and did not ever reach the late larval or pupal

TABLE 3

Reversal of the *Su(DTS)* suppression of DTS lethality of *Pros*β*2*^{1} by a *Pros25*$^{+}$ transgene

F_1: genotype	No.
Cross (at 29°C): *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/TM3, Sb p*^{p} *e* × *w; +/+; Pros*β*2*^{1} *st tra in p*^{p}*/TM6B, Tb e ca*	
A. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/TM6B, Tb e ca*	80
B. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; st ri p*^{p} *Su(DTS) e ca/Pros*β*2*^{1} *st tra in p*^{p}	0
C. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; Pros*β*2*^{1} *st tra in p*^{p}*/TM3, Sb p*^{p} *e*	0
D. *w; P{Pros25-2.0 KB, w*$^{+}$*}10A/+; TM3, Sb p*^{p} *e/TM6B, Tb e ca*	6
E. *w; +/+; st ri p*^{p} *Su(DTS) e ca/TM6B, Tb e ca*	98
F. *w; +/+; st ri p*^{p} *Su(DTS) e ca/Pros*β*2*^{1} *st tra in p*^{p}	108
G. *w; +/+; Pros*β*2*^{1} *st tra in p*^{p}*/TM3, Sb p*^{p} *e*	0
H. *w; +/+; TM3, Sb p*^{p} *e/TM6B, Tb e ca*	3

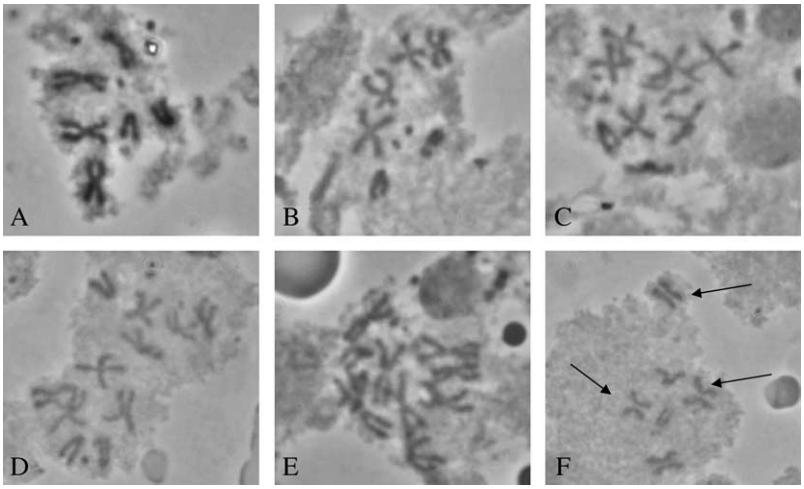

FIGURE 3.—Metaphase spreads from larval brains of (A) *w; Pros25^{SuDTS}/Pros25^{SuDTS}*, showing a normal female karyotype; (B) *w/Y; Pros26^{1}/+*, showing a normal male karyotype; and (C–F) *w; Pros*β*2^{1}/+*, showing aneuploidy (C–E) and precocious chromatid separation (F) (arrows).

stages. This suggests that *Pros25^{SuDTS}* is not a complete loss-of-function allele. When *Df(3R)T-32/MKRS* flies were crossed to *Pros26^{1}/TM6B, Tb e ca* or *Pros*β*2^{1}/TM6B, Tb e ca* and progeny reared at 29°, there were some non-Tubby survivors, although the deficiency was not as effective at suppressing the DTS lethality of these mutants as the *Pros25^{SuDTS}* mutant. This suggests that reducing the amount or activity of the Pros25 subunit can somehow alleviate the defects caused by the DTS proteasome mutants.

Effect of *Pros25^{SuDTS}* on proteasome function: To investigate the mechanism of how *Pros25^{SuDTS}* suppresses the DTS lethality of *Pros26^{1}* and *Pros*β*2^{1}*, the effect of these three mutants on proteasome function was assessed, using an assay adapted from previous experiments of SCHWEISGUTH (1999), who used immunofluoroscopy to monitor the degradation of a known target of the ubiquitin–proteasome pathway, the Drosophila Nintra protein. Here, we created a heat-shock-inducible, unstable EGFP by joining a portion of Notch containing its PEST degradation signal (WESLEY and SAEZ 2000) to the carboxy-terminal region of EGFP and placing the fusion gene downstream of the heat-shock promoter in the transformation vector, pCasPer-hs (THUMMEL and PIRROTTA 1992). When transgenic larvae are subjected to a 30-min heat shock at 37°, the EGFP-Nintra reporter protein is ubiquitously expressed, reaching peak fluorescence within 1 hr and then steadily diminishing until it is only weakly detectable after 4 hr (Figure 4, A–C). In contrast, a hs-EGFP transgene, lacking the Notch sequences, produces heat-shock-inducible green fluorescence that is notably more stable and is easily detected 4 hr post-heat shock (Figure 4, D–F). To examine the effects of dominant proteasome mutants on the degradation of heat-shock-induced EGFP-Nintra, the mutant subunits were ectopically expressed in a spatially restricted manner in larval wing discs using the UAS/GAL4 system. In these experiments, a *ptc*-GAL4 "driver" was used [*i.e.*, *P{w$^{+mW.hs}$=GawB}ptc$^{559.1}$*], which expresses GAL4 in cells along the anterior/posterior boundary of the wing disc (JOHNSON *et al.* 1995). If expression of a proteasome mutant subunit inhibits proteasome function (*i.e.*, if it acts in a dominant-negative manner) then the EGFP-Nintra reporter protein should be stabilized, and fluorescence will appear brighter in those cells expressing GAL4. Consistent with the results of SCHWEISGUTH (1999) who looked at the effect of Pros26^{1} on Notch-intracellular protein stability, EGFP-Nintra was notably stabilized by the expression of Pros26^{1} (Figure 4G, arrow). A similar inhibition of EGFP-Nintra degradation was seen with expression of Prosβ2^{1} (Figure 4H, arrow). These results confirm that both of these DTS mutants act in a dominant-negative manner to inhibit proteasome activity. Similar ectopic expression of wild-type proteasome subunits, *e.g.*, Pros25 or Pros29 (*Pros29* encodes the α3 subunit of the 20S proteasome) (Figure 4, I and J) or of an unrelated control protein, *Escherichia coli* β-Gal (not shown), had no effect on the stability of EGFP-Nintra, demonstrating that the effect of the DTS mutants is not due to protein overexpression, *per se*, but is specific to these mutant subunits.

When the Pros25^{SuDTS} mutant alone is expressed in this system, there is no detectable effect on the degradation of EGFP-Nintra (Figure 4K). The simultaneous expression of Pros25^{SuDTS} and Prosβ2^{1}, however, restores normal degradation of EGFP-Nintra (Figure 4L), indicating that the mutant Pros25 subunit acts to reverse the dominant-negative effect of Prosβ2^{1} on proteasome function. While this result is not surprising, it is significant in that it demonstrates a direct effect of the suppressor mutant on the function of a gene that it suppresses. This result also demonstrates that the *Pros25^{SuDTS}* allele is not acting as an amorph or a hypomorph, since its forced expression has a dominant effect on proteasome function in this assay. A simple loss-of-function allele would not be expected to act in such a dominant manner in this system.

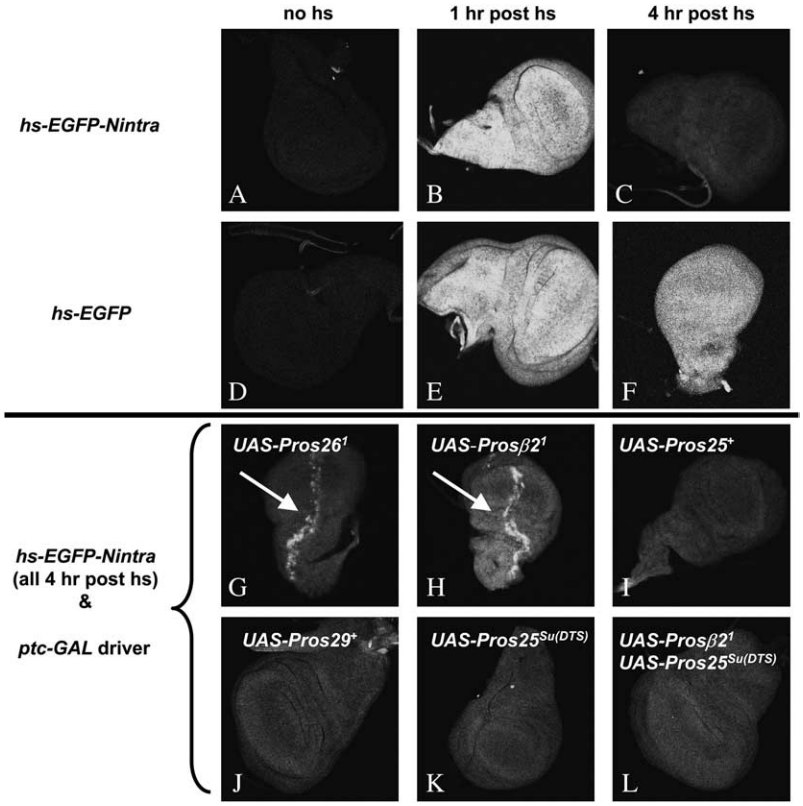

FIGURE 4.—Effects of mutant proteasome subunits on the degradation of a heat-shock-inducible green fluorescent protein reporter in larval wing discs. (A–C) Wing discs from *w P{hs-EGFP-Nintra, w⁺}15A(X)* larvae. (A) No heat shock, (B) 1 hr post-heat shock, (C) 4 hr post-heat shock. (D–F) Wing discs from *w; P{hs-EGFP, w⁺}25A(3).* (D) No heat shock, (E) 1 hr post-heat shock, (F) 4 hr post-heat shock. (G–L) Wing discs, 4 hr post-heat shock from (G) *w P{hs-EGFP-Nintra, w⁺}15A(X)/P{UAS-Pros26¹, w⁺}11A(X); P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/+*, (H) *w P{hs-EGFP-Nintra, w⁺}15A(X); P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/+; P{UAS-Prosβ2¹, w⁺}2B(3)/+*, (I) *w P{hs-EGFP-Nintra, w⁺}15A(X); P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/P{UAS-Pros25⁺, w⁺}4A(2)*, (J) *w P{hs-EGFP-Nintra, w⁺}15A(X); P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/P{UAS-Pros29⁺, w⁺}1(2)*, (K) *w P{hs-EGFP-Nintra, w⁺}15A(X);P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/P{UAS-Pros25SuDTS, w⁺}2A(2)*, and (L) *w P{hs-EGFP-Nintra, w⁺}15A(X); P{w⁺ᵐᵂ.ʰˢ=GawB}ptc⁵⁵⁹.¹}/P{UAS-Pros25SuDTS, w⁺}2A(2); P{UAS-Prosβ2¹, w⁺}2B(3)/+*. The arrows point to the stabilization of EGFP-Nintra in the cells expressing the *Pros26¹* or *Prosβ2¹* mutants.

DISCUSSION

X-ray crystallographic studies of yeast and mammalian proteasomes have shown that the eukaryotic 20S core is highly conserved in its overall structure (GRÖLL *et al.* 1997; UNNO *et al.* 2002). Three of the β-type subunits (β1, β2, and β5) are catalytic with the mechanism involving their amino-terminal threonines that face inward toward a large central cavity. Several of the β-type subunits, including all catalytic subunits, are synthesized as proproteins that undergo autocatalytic processing following their assembly into the 20S complex (SCHMIDTKE *et al.* 1996). The noncatalytic β-type subunits do not have N-terminal threonines; however, they can have effects on proteasome activity through their structural role in forming the degradative chamber where they can physically interact with the substrates' side chains (GRÖLL *et al.* 2000). The α-type subunits in the two outer rings have no direct catalytic function, although they do assist in the ordered assembly of the 20S particle (SCHMIDTKE *et al.* 1996), and they may play regulatory roles. For example, the α-rings form antechambers through which unfolded polypeptides must pass, and it is possible that there are regulatory interactions between the α-subunits and the substrate as it awaits entry into the innermost chamber. The α-subunits also interact directly with the 19S cap and with an alternative regulator called the 11S complex, and they can affect the subcellular distribution of proteasomes via nuclear localization signals present on α1, α2, α3, and α4 (UNNO *et al.* 2002). The 20S proteasome is not ordinarily an open cylinder, but is sealed off at each end by the N-terminal tails of α2, α3, and α4 and an internal loop of α5 (GRÖLL *et al.* 2000; UNNO *et al.* 2002). This gate must be opened before a substrate can enter the core, a task performed by regulatory complexes such as the 19S cap. Although these general features of the structure and function of the proteasome are known, the exact roles of each of the individual subunits are less well understood. For example, it is not known if the α2 subunit encoded by *Pros25* has any special function that is distinct from the roles of α-type subunits in general.

The genetic and biochemical properties of the *Prosβ2¹* and *Pros26¹* mutants suggest that they encode abnormal β2 and β6 subunits that incorporate into proteasome particles and interfere with their function (SAVILLE and BELOTE 1993; COVI *et al.* 1999; SCHWEISGUTH 1999; SMYTH and BELOTE 1999). This "poison subunit" hypothesis explains how these mutants act in a dominant-negative fashion (HERSKOWITZ 1987). However, the exact mechanism by which these abnormal subunits are interfering with proteasome activity is not known. Although the Drosophila proteasome structure has not been solved, the high degree of structural similarity between the yeast and bovine structures suggests that the fly 20S particle does not differ in its overall structure from those two. Using the bovine proteasome as the model, in *Prosβ2¹* there is a replacement in the β2 subunit of a highly conserved glycine by an arginine at

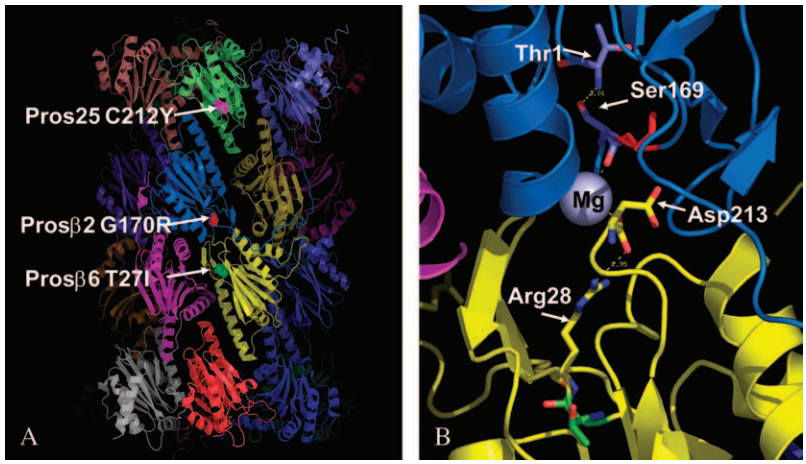

FIGURE 5.—X-ray structure of the bovine 20S proteasome as determined by UNNO *et al.* (2002). (A) Ribbon diagram of the 20S proteasome showing the relative positions of the amino acid substitutions in the Drosophila *Pros25*^{SuDTS} (α2 subunit), *Pros*β*2*^{1} (β2 subunit), and *Pros26*^{1} (β6 subunit) mutants. The α2 subunit is light green, β2 is blue, and β6 is yellow. The corresponding sites of the *Pros25*^{SuDTS}, *Pros*β*2*^{1}, and *Pros26*^{1} amino acid substitutions are shown in magenta, red, and green, respectively. (B) Spatial relationship between the Gly170 of β2 (red) and the Thr27 of β6 (green). Also shown is the Mg^{2+} ion that forms a bridge between Ser169 of β2 and Asp213 of β6. The N-terminal active site threonine (Thr1) of β2 is 3.01 Å away from Ser169. Arg28 of β6 is 2.95 Å from Asp213. All of these residues are conserved between the bovine and fly proteasomes.

amino acid position 170 (or 209 before autocatalytic processing). This is in a loop between α-helix four and β-sheet nine and is located near the active site of β2 in the three-dimensional (3D) structure (Figure 5). This loop may be critical for stabilizing the interaction between the β2 and β6 subunits in adjacent rings. For example, the carbonyl oxygen of Ser169 in β2 interacts via a magnesium ion bridge with the C-terminal aspartate (Asp213) of the β6 subunit. It is likely that the substitution of a bulky arginine for Gly170 would reduce the flexibility of the loop and interfere with Mg^{2+} binding. This might not only affect the stability of the β2–β6 interaction but it could also very well have a direct effect on catalytic function, since the γ-hydroxyl side chain of the highly conserved Ser169 of β2 is only 3.0 Å from the amino group of its active site threonine (Figure 5B). This is close enough to provide a stabilizing influence on its positioning via hydrogen bonding. A shift in the position of Ser169 caused by the Gly170Arg substitution in *Pros*β*2*^{1} could thereby interfere with the active site of the β2 subunit. This shift might be expected to occur more readily at elevated temperature, thus explaining the temperature sensitivity of this mutant.

In *Pros26*^{1}, there is a threonine to isoleucine substitution at position 27 (or position 47 before processing) of the β6 subunit. This change occurs in a highly conserved loop between β-sheets two and three, immediately adjacent to an arginine (Arg28) that forms a salt bridge with the C-terminal carbonyl of the Asp213, mentioned above as important for Mg^{2+} binding (Figure 5B). This Thr27Ile substitution may cause a subtle structural shift that alters the position of Arg28, which could then indirectly affect the Mg^{2+} binding pocket and interfere with the β2–β6 interaction, or it might inhibit the catalytic function of β2 as described above. The importance of this spatial relationship among a C-terminal aspartate, a magnesium ion, Ser169, and the active site threonine (Thr1) is highlighted by the fact that a similar type of arrangement is seen in the structure surrounding the active site of the β5 subunit. In that case a magnesium ion bridges an interaction between the C-terminal aspartate of β3 and Ser169 of β5, with the side-chain hydroxyl of Ser169 interacting with the amino group of the active site threonine of β5. The significance of Mg^{2+} is supported by results of *in vitro* assays in which proteasome activity is stimulated by the presence of magnesium ions (PEREIRA *et al.* 1992).

In *Pros25*^{SuDTS} the Cys212Tyr replacement occurs at the end of β-sheet eight in a position that is partially surface exposed (Figure 5A). The results described here show that the mutant subunit can act in a dominant manner to rescue the temperature-sensitive lethality of both *Pros*β*2*^{1} and *Pros26*^{1}, and in the case of *Pros*β*2*^{1} this has been shown to be associated with a restoration of proteasome functional activity. This suppression is not likely due to a direct compensating effect of the Cys212Tyr replacement in Pros25^{SuDTS} interacting with the Gly170Arg substitution in Prosβ2^{1} or the Thr27Ile mutation in Pros26^{1}, given their relatively remote positions in the predicted 3D structure (Figure 5A). It seems more likely that the mutant α2 subunit in *Pros25*^{SuDTS} is indirectly counterbalancing the inhibitory effects of the *Pros*β*2*^{1} and *Pros26*^{1} mutations in β2 and β6, respectively. For example, it may be that the Cys212Tyr replacement in the α2 subunit results in a more effective movement of polypeptides through the proteasome and that this gain in proteasome efficiency helps overcome the slowdown in proteolysis caused by the β-subunit mutations. If this is true, it is not obvious what the mechanistic basis for this is. The position of Cys212Tyr appears too far from the N-terminal tail in the 3D structure to affect the gating of the 20S proteasome, and it is not exposed to the internal antechamber, so it would not be expected to interact directly with proteasome substrates. It is possible that the substitution of the cysteine with a bulky aromatic tyrosine might have an effect on the overall folding of the α2 subunit, and this change in tertiary structure could have

unpredictable effects. Since the α2 subunit has been shown to interact directly with the 11S proteasome regulator (Kania *et al.* 1996) it is also conceivable that the mutant α2 subunit might affect proteasome activity through an interaction with this or the 19S cap.

Whatever the mechanism, the suppression of a proteasome mutant by mutation in a gene encoding another proteasome subunit has a precedent. In *S. cerevisiae*, the *crl3-2* mutant is a temperature-sensitive lethal allele of the gene encoding the Rpt6 subunit of the 19S regulatory cap (Gerlinger *et al.* 1997). A dominant suppressor of *crl3-2*, called *SCL1-1*, was isolated and subsequently found to represent a mutant of the gene encoding the α1 subunit of the 20S core (Balzi *et al.* 1989; Gerlinger *et al.* 1997). Biochemical analyses of proteasome function showed that while the *crl3-2* mutant had defective proteasome activity, in *crl3-2 SCL1-1* double mutants proteasome function is restored (Gerlinger *et al.* 1997). As is the case with our study, the mechanistic basis of this suppression is unknown.

Regardless of how $Pros25^{Su(DTS)}$ suppresses both $Pros26^{1}$ and $Pros\beta2^{1}$, the results described here demonstrate that this type of suppressor screen might be used to efficiently isolate new proteasome mutants. Because the suppression phenotype, *i.e.*, viability, is very easy to identify in this screen, it should be feasible to carry out such a screen on a large scale to identify additional proteasome mutants. By using different combinations of the dominant-negative conditional mutants and dominant suppressors, in conjunction with the UAS/GAL4 system, it may be possible to finely tune proteasome malfunction in a targeted manner. Such a system might be useful in cases where drastic effects on proteasome function might be too harmful to assess the role of the ubiquitin–proteasome pathway on the process of interest.

To date, only a few proteasome mutants have been identified in Drosophila, and most are severe loss-of-function alleles that act as recessive, embryonic, or early larval lethals. For example, recessive lethal alleles of the Prosβ2, Prosβ5, and Prosβ6 20S proteasome subunit genes have been isolated and they behave as amorphs, or severe hypomorphs, with lethality occurring soon after hatching from the embryo (Saville and Belote 1993; Smyth and Belote 1999; S. Eaton, personal communication). It is likely that maternal contribution of proteasome subunits or their mRNAs prevents these mutants from exhibiting an earlier lethal phase (Ma *et al.* 2002). A null allele of the gene encoding the 19S regulatory particle subunit Rpn10 has a pupal lethal period (Szlanka *et al.* 2003). Recent efforts of large-scale gene disruption projects have resulted in the identification of transposon insertion alleles of a few other proteasome subunits, but they have yet to be studied in detail.

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