



Localisation of 26S proteasomes with different subunit composition in insect muscles undergoing programmed cell death

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Abstract

The 26S proteasome is a large multisubunit complex involved in degrading both cytoplasmic and nuclear proteins. We have investigated the subcellular distribution of four regulatory ATPase subunits (S6 (TBP7/MS73), S6' (TBP1), S7 (MSS1), and S10b (SUG2)) together with components of 20S proteasomes in the intersegmental muscles (ISM) of *Manduca sexta* during developmentally programmed cell death (PCD). Immunogold electron microscopy shows that S6 is located in the heterochromatic part of nuclei of ISM fibres. S6' is present in degraded material only outside intact fibres. S7 can be detected in nuclei, cytoplasm and also in degraded material. S10b, on the other hand, is initially found in nuclei and subsequently in degraded cytoplasmic locations during PCD. 20S proteasomes are present in all areas where ATPase subunits are detected, consistent with the presence of intact 26S proteasomes. These results are discussed in terms of heterogeneity of 26S proteasomes, 26S proteasome disassembly and the possible role of ATPases in non-proteasome complexes in the process of PCD. *Cell Death and Differentiation* (2000) 7, 1210–1217.

Keywords: programmed cell death; tobacco hornworm; *Manduca sexta*; proteasome regulatory ATPase subunit; muscle degradation; immunogold method

Abbreviations: ISM, abdominal intersegmental muscles; PCD, programmed cell death; AAA, ATPases associated with diverse cellular activities; EM, electron microscopy

Introduction

The intersegmental muscles (ISMs) of the tobacco hornworm, *Manduca sexta* are a particularly useful model for examining the biochemical events mediating programmed cell death (PCD). The ISMs are retained until 3 days before adult

eclosion at which point they begin to atrophy as a result of a decline in the circulating titre of the insect moulting hormone (20-hydroxyecdysone). Atrophy involves the loss of muscle mass, without change in either morphology or physiological function. A further decline in ecdysteroids just before emergence commits the ISMs to die during the 36 h following adult eclosion.¹

Muscle proteins to be eliminated are labelled by ubiquitin and digested by a multicatalytic enzyme complex, the 26S proteasome.² This proteolytic machine consists of a 20S proteasome core and two regulatory 19S 'caps', one at either end of that core. Two juxtaposed rings of seven β -type subunits flanked on both the top and bottom by a ring of seven α -type subunits form the barrel-shaped 20S core complex. Together, these four rings enclose three inner compartments, two antechambers and one central proteolytic chamber. Each regulatory 19S cap contains a 'base' and a 'lid' complex. The 'base' complex, which directly contacts an α -type ring of the 20S core, is currently thought to comprise all six ATPases (S4, S6, S6', S7, S8, S10b), and the two largest subunits (S1, S2), as well as S5a, the ubiquitin binding subunit. The 'lid' complex consists of eight subunits and is necessary for degradation of ubiquitinated target proteins. Several functions have been proposed for the ATPases. The hydrolysis of ATP is supposed to promote the assembly of 26S proteasomes. The ATPases could have a role in the gating of the translocation channel on the 20S particle. Substrate proteins are possibly bound and unfolded by the ATPases. The ATPases might assist in the translocation of unfolded substrate proteins into the central proteolytic chamber of the 20S proteasome.³ Consistent with these proposals the 'base' complex of the 19S cap has recently been shown to have chaperone-like activity.^{4,5}

Examples of programmed cell death are sometimes classified into two groups.⁶ Type I is characterised by early nuclear collapse and DNA fragmentation and typified by apoptosis in cells of haematopoietic origin. Type II, often seen in post-mitotic cells and during metamorphosis, is characterised by early consumption of the bulk of the cytoplasm whereas nuclear collapse is a late event. In type I cell death, the ubiquitin-proteasome system impinges on key molecules which regulate apoptosis, and proteasome inhibitors can either induce or block cell death depending on cell type and conditions.^{7,8} In type II cell death, typified by loss of ISM in *Manduca sexta* elimination of muscle cytoplasm is brought about by a combination of lysosomal and proteasomal activity.^{1,9,10}

Recent studies in *Manduca sexta* have shown specific developmental changes in the regulatory subunits of the 26S proteasome in ISM during PCD.^{11,12} However, there has as yet been no detailed electron microscopic analysis of the sub-cellular localisation of 26S proteasomes and

specific subunits during the developmentally programmed neuromuscular cell death in *Manduca*. Moreover, no one studied possible changes in subunit composition in proteasomes of a tissue during differentiation or cell death. We have therefore determined the subcellular distribution of four proteasome regulatory ATPase subunits (S6, S6', S7 and S10b, named according to¹³) and the core 20S particle in ISM during PCD. Immunogold electron microscopic studies using affinity purified antisera revealed a diverse localisation pattern. While S7 and 20S were found both in nuclear and cytoplasmic areas and even outside the muscle fibres in degraded material, S6 had mainly a nuclear localisation and S6' was found only outside fibres. Interestingly, during development S10b changed its location: it was in the nucleus first and moved to the outside later. These results are discussed in terms of the possible roles of populations of 26S proteasomes with different ATPase regulators, of non-proteasomal complexes containing ATPases, and of dissolution of 26S proteasomes in ISM during PCD.

Results

Testing the specificity of antibodies

Immunoblots of protein extracts of stage 0 and stage 7 muscles incubated with affinity purified antibodies against regulatory ATPase subunits show single bands of the expected molecular mass: S6 and S6' \cong 47 kDa, S7 \cong 45 kDa and S10b \cong 43 kDa (Figure 1) and confirm the marked increase in amount of each ATPase between stages 0 and 7 that were observed previously.^{11,14} These affinity purified antisera have been used to perform immunogold studies.

Distribution of proteasome ATPase subunits in ISM before the onset of PCD (stage 0)

Intersegmental muscle fibres of stage 0, before the 20-hydroxyecdysone commitment peak,¹⁵ are intact showing no signs of degradation (Figure 2). Immunogold investigation of the subcellular localisations of the four studied regulatory ATPase subunits and the 20S proteasome core subunit revealed the pattern which is summarised in Table 1. Dense heterochromatin areas of nuclei are decorated with gold particles corresponding to three regulatory ATPase subunits (S7, Figure 2a; S10b, Figure 2b; S6, not shown) and the 20S proteasome core subunit (Figure 2c). Nucleoli and areas of myofibrils and cytoplasm are free of labelling in the case of S6 (not shown) and S10b ATPases (Figure 2b). However, gold particles corresponding to S7 ATPase (Figure 2a) and 20S proteasome core subunit (Figure 2c) are also found above cytoplasmic areas, contractile apparatus and even degraded material outside intact fibres. Two thirds of S6' ATPase specific gold particles are situated above cytoplasm and the remaining one third above remnants of degraded fibres (Table 1). Since 20S proteasome core subunits can be detected in all those areas where the regulatory ATPase subunits occur (Table 1) it is probable that significant amounts of the detected ATPase subunits (S6, S6', S7 and S10b) are present as

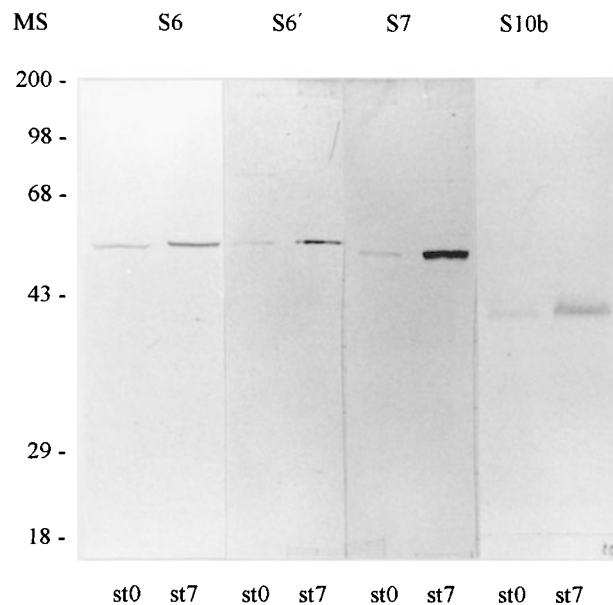


Figure 1 Immunodetection of proteasome regulatory subunits in ISM extracts. Proteins from stage 0 and stage 7 ISM (10 μ g per lane) were separated by SDS/PAGE and immunoblotted with affinity-purified antibodies specific to proteasome ATPase subunits (S6, S6', S7 and S10b). The positions of molecular-mass markers and their size in kDa are indicated on the left

integral components of the 26S proteasome. Immunogold labelling is not very intense at stage 0 in either the case of proteasome core or ATPase subunits indicating that muscle degeneration has not begun, not even in the muscles which become committed to die.

Distribution of proteasome ATPase subunits in ISM during PCD (stages 7 and 8+15 h)

The ISMs are retained until 3 days before adult eclosion at which point they begin to atrophy after the 20-hydroxyecdysone titre begins to decline. Atrophy involves the loss of muscle mass fibre by fibre, without change in contractility of the whole piece of muscle. As development proceeds more and more fibres undergo cell death. Fibre nuclei become pycnotic and still remain even when sarcoplasm and contractile elements completely lose integrity. Immunogold investigation shows that the distribution of S6' and S10b ATPase regulatory subunits changes during PCD while that of other subunits remains essentially the same (Table 1). Dense heterochromatin areas of nuclei are decorated with gold particles corresponding to S6 ATPase during PCD (Figures 3a and 4a). S6' ATPase specific gold particles can only be seen above remnants of degraded fibres (Figure 3b, Table 1), the amounts of which increase from stage 0 to stage 7 and further to stage 8+15 h (not shown). At stage 7, gold particles corresponding to S10b ATPase appear outside intact fibres above degraded material while they also remain observable above intact nuclei as well (Figure 3c). At the final stage S10b labelling can be detected only outside viable fibres and is no longer present in the nuclei (Figure 4b). Gold particles

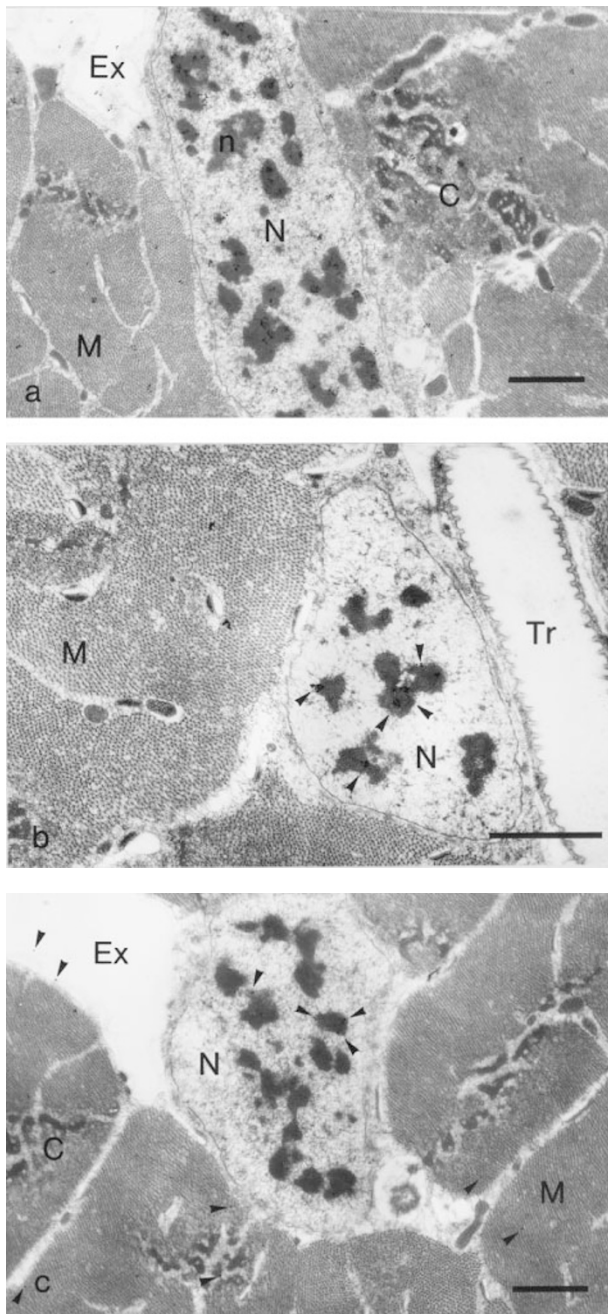


Figure 2 Immunolocalisation of proteasome subunits in ISM before the onset of PCD (stage 0). Heterochromatic areas of nuclei, cytoplasmic areas, contractile apparatus and also degraded material are decorated with gold particles corresponding to S7 (MSS1) ATPase (a). Heterochromatic areas of nuclei are labelled with gold particles corresponding to S10b (SUG2) ATPase (arrowheads) (b). Heterochromatic areas of nuclei, cytoplasmic areas, contractile apparatus and also degraded material are decorated with gold particles corresponding to 20S proteasome core subunits (arrowheads) (c). N, nucleus; n, nucleolus; C, cytoplasm; M, myofibrils; Ex, extracellular space; Tr, trachea. Bars 1 μ m

corresponding to S7 ATPase and 20S proteasome core subunit are distributed above dense heterochromatin areas of nuclei, cytoplasmic areas, contractile apparatus and also

Table 1 Summary of the localisation of proteasome subunits in *Manduca* intersegmental muscles

	Stage 0			Stage 7			Stage 8+15		
	NU	CP	EX	NU	CP	EX	NU	CP	EX
S6	74	22	4	87	8	5	80	15	6
S6'	7	60	34	2	2	96	1	2	97
S7	46	38	16	46	35	19	52	22	26
S10b	87	13	0	40	8	52	1	8	91
20S	48	39	13	25	48	27	57	16	27

The table shows percentage distribution of gold particles corresponding to proteasome subunits among compartments (NU, nuclear; CP, cytoplasmic; EX, extracellular) at studied developmental stages. For each antigen there was a significant rise in the total number of gold particles per unit area between stage 0 and stage 7, with a smaller change thereafter either up or down. Values were calculated upon cumulated data of 3–4 EM micrographs (magnification 8,000 \times). Between 100 and 1000 particles were counted for each antigen at each stage

degraded material outside intact fibres during PCD (stage 7 and 8+15 h) as they were in stage 0 (20S at stage 7, Figure 3d; others are not shown).

Controls, where the first, ATPase subunit specific antibodies were omitted, are free of labelling (not shown). In the case of 20S proteasome core subunit specific antibody pre-immune serum (serum taken from the rabbit before the immunisation process) was available. Sections where the pre-immune serum has been used instead of first antibody also show no labelling on fibres (not shown). In order to determine whether the ATPase subunits localised by immunogold EM are integral components of 26S proteasomes, extracts of stage 7 ISM were subjected to glycerol gradient analysis. (Remnants of ISM at stage 8+15 h are so minute and delicate that it is impossible to collect sufficient material for a similar analysis with tissue from this stage.) Glycerol gradient fractions corresponding to 26S proteasomes and to bulk soluble ISM protein were analysed by semi-quantitative immuno-dotblot against the ATPase antibodies. The results (not shown) indicate that 94–99% of each ATPase in stage 7 ISM is associated with the 26S proteasome, and corroborate more extensive studies which we have previously published, showing that up to 5% of each ATPase sediments through glycerol gradients independently of the 26S proteasome.^{11,14} The implications of these results are considered below.

Discussion

We have clearly demonstrated that in *Manduca* ISM, 20S core subunits are localised in the same compartments (nucleus, cytoplasm, contractile apparatus, disrupted fibres) as regulatory ATPases of the proteasome (Table 1). While it cannot be proven that these ATPases are in all cases associated with 26S proteasomes without *in vitro* pull down experiments, or at the very least, dual particle gold labelling immuno electron microscopy, other evidence suggests that the majority of ATPases are indeed components of 26S particles. Our previous work has analysed extracts from stage 0 and stage 7 ISM by glycerol gradient sedimentation and quantitative Western blotting and shown that while the levels of both 20S components and ATPases (including S6, S6', S7

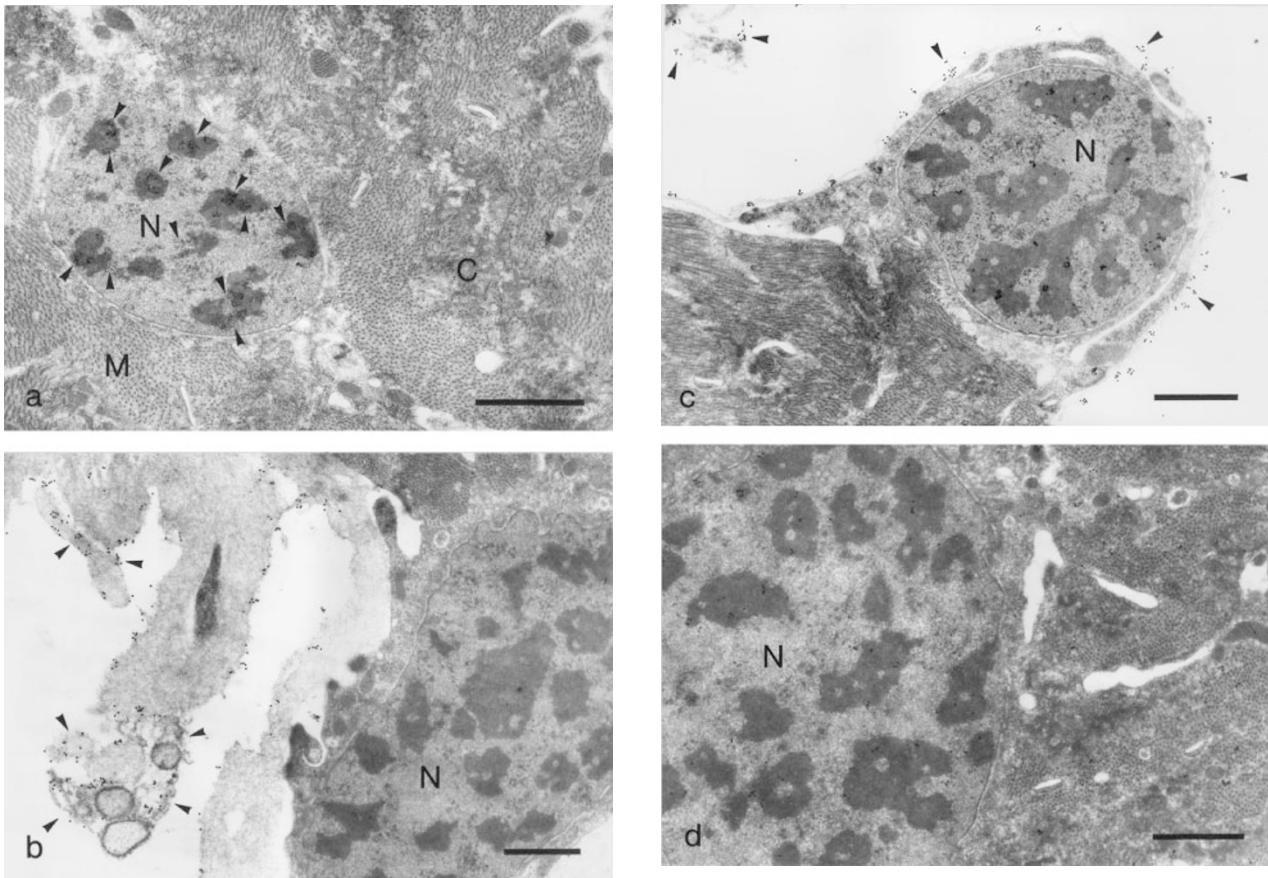


Figure 3 Immunolocalisation of proteasome subunits in ISM during PCD (stage 7). Heterochromatic areas of nuclei are heavily decorated with gold particles corresponding to S6 (MS73) ATPase subunit (arrowheads) (a). Degraded material of disintegrated fibres (arrowheads) between intact ones is thoroughly labelled with gold particles corresponding to S6' (TBP1) ATPase (b). S10b (SUG2) ATPase subunits can be detected outside intact fibres above degraded material (arrowheads) while they also remain observable above intact nuclei (c). Heterochromatic areas of nuclei, cytoplasmic areas, contractile apparatus and also degraded material are decorated with gold particles corresponding to 20S proteasome core subunits (d). N, nucleus; C, cytoplasm; M, myofibrils, Bars 1 μ m

and S10b) increase substantially during neuromuscular cell death, at both stage 0 and stage 7, more than 90–95% of each ATPase is associated with the 26S proteasome.^{11,14} These figures refer to total ISM extract and do not preclude small amounts of ATPase in particular subcellular locations which are not associated with the 26S particle. The current observations, in which four different regulatory proteasome ATPases were found not to co-localise with each other, imply either (a) that 26S proteasomes exist in ISM without the full complement of the six different ATPases (perhaps including more than one molecule of a particular ATPase per 19S cap), or (b) that non-proteasomal complexes are present in ISM without the full complement of ATPases. The first suggestion runs counter to current popular views on 26S proteasome structure, based on a definitive analysis in yeast¹⁶ but remains a real possibility for more complex multicellular eukaryotes in the light of the present work. The second suggestion is true in the case of ISM to the extent that we have recently demonstrated the presence of a 220 kDa activator of the proteasome which contains S6' and S10b, but not S6 or S7.¹⁴ This activator is present in both stage 0 and stage 7 ISM and increases in amount during development in a similar manner to the 26S proteasome. It represents no more than 5% of the

S10b in ISM compared to the 26S proteasome. The activator is not identical but is related to the modulator complex described in mammalian erythrocytes.¹⁷ It is noteworthy that in the current EM analysis, S6' and S10b show distinct localisations except at stage 8+15 h, suggesting that the 220 kDa activator does not make a significant contribution to the immunogold observations. Several of the proteasomal ATPases were first discovered by their interaction with transcription factors (S8, S10b,¹⁸) or with viral proteins (S6, S6',¹⁹) before their role as components of the 26S proteasome was demonstrated.^{20,21} However, our previous analysis of soluble extracts from total ISM tissue failed to detect any complexes containing S6, S6', S7 or S10b except the 26S proteasome or 220 kDa activator.

In early ultrastructural studies of the degeneration of Lepidopteran ISM it was observed that autophagy is responsible for the selective destruction of mitochondria, glycogen particles, ribosomes, and other organised sarcoplasmic structures.⁹ However, these authors found that the dissolution of the myofilaments is independent of lysosomal activity. This is in agreement with our results since we found gold particles corresponding to proteasome regulatory subunits and 20S core particles above nuclei,

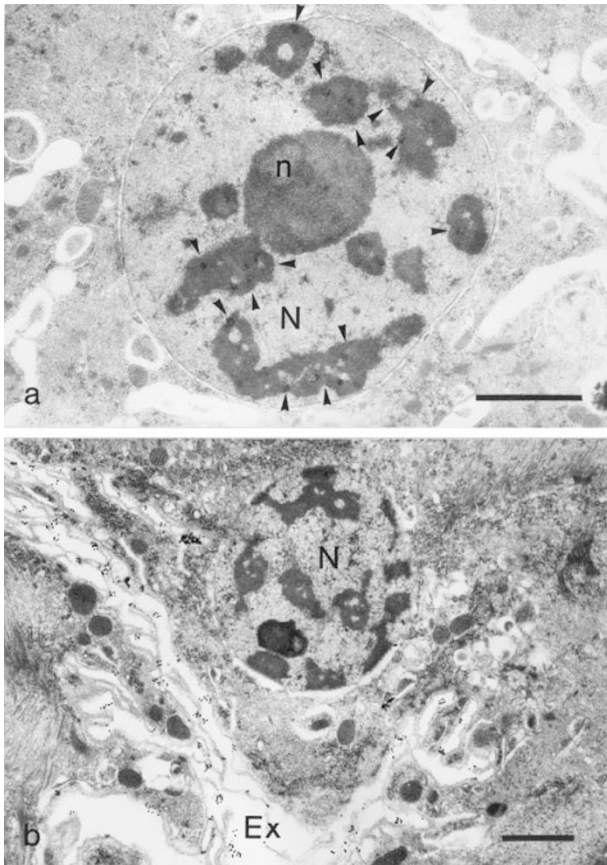


Figure 4 Immunolocalisation of proteasome subunits in ISM during PCD (stage 8+15 h). Heterochromatic areas of nuclei are decorated with gold particles corresponding to S6 (MS73) ATPase subunit (arrowheads) (a) Note nucleolus (n) is free of labelling. S10b (SUG2) ATPase subunits can be detected only outside viable fibres and not in the nuclei of ISM (b). Note pycnotic nuclei in stage 8+15 h. N, nucleus; n, nucleolus; Ex, extracellular space. Bars 1 μ m

contractile apparatus and cytoplasmic areas free of organelles. Another interesting feature of the programmed degeneration of ISM is that nuclei become pycnotic but remain intact in degraded fibres while the contractile apparatus is digested.⁹ All ATPases that we have analysed, and 20S core particles appear to increase in abundance between stage 0 and stage 7 (Table 1), in agreement with our previous data.^{11,14} Detailed biochemical analyses of stage 8+15 h ISM have not been made, so that we cannot assess possible dissociation of ATPases from the proteasome at this stage. However, the current observations demonstrate the release of proteasome particles from disintegrating cells together with partially degraded muscle fibres. The predominance of S6' and S10b ATPases in this extracellular material may reflect their relative resistance to proteolytic degradation compared to other proteasomal components (e.g. S6 and S7). About three days before adult emergence, the ISMs begin to atrophy, which results in dramatic loss of muscle weight without significant changes in the physiological properties of the muscle. This phenomenon shows that the degrada-

tion process proceeds fibre by fibre, one fibre degenerates while neighbouring ones remain functional but also committed to die. Motoneurons in the fourth abdominal ganglion innervating these ISMs degenerate in the same fashion.²² The destruction of motoneurons starts 12 h after eclosion but intact neurons can be observed even 20 h later when there are still surviving, contractible muscle fibres i.e. indicating again that cells die one by one. While the average number of intact fibres or neurons decrease continuously during PCD an individual fibre or neuron can survive until the end of the process.

There are several investigations where nuclear localisation of proteasomes has been demonstrated (see²³). According to quantitative immunoblot analysis of purified rat liver subcellular fractions, proteasomes were mainly found in the cytosol but were also present in the purified nuclear and microsomal fractions.²⁴ In the nuclei, proteasomes were soluble or loosely attached to the chromatin. In budding yeast (*Saccharomyces cerevisiae*) microscopic analysis revealed that 20S as well as 19S subunits of proteasome are accumulated mainly in the nuclear envelope–endoplasmic reticulum network.²⁵ In fission yeast (*Schizosaccharomyces pombe*) immunofluorescence microscopy shows a striking localisation pattern whereby proteasomes are found predominantly at the nuclear periphery, both in interphase and throughout mitosis, whereas during meiosis dramatic relocalisation of proteasomes within the nucleus occurs.²³ These findings strongly imply that proteasomes have important proteolytic roles in the nucleus by degrading regulatory proteins. Indeed, Lo and Massagué²⁶ have recently provided direct evidence for such a role of nuclear 26S proteasomes. They showed that Smad2, a transcription factor responsive to TGF β signalling is degraded in the nucleus by selective ubiquitination and proteasome action. In the rat central nervous system (CNS) proteasomes are ubiquitously distributed and localised primarily in the nuclei of neurons.²⁷ Proteasomes may participate in the mechanism of maintenance of long-term potentiation (LTP) by the degradation of inducible transcription factors (NF κ B/I κ B, fos, jun, p53) modulating LTP, via ubiquitin-dependent or ubiquitin-independent pathways.²⁷ Preliminary immunohistochemical results show ubiquitinated protein accumulation in nuclei of degenerating neurons in sections of Creutzfeldt-Jacob disease infected human brain (L. László, personal communication). Ubiquitinated protein accumulation implies that the neurons under stress try to degrade their misfolded or damaged nuclear proteins via the ubiquitin-proteasome pathway. This would mean that the ubiquitin-proteasome system is activated under permanent stress during neurodegradation similarly to the process of developmentally programmed cell death.

In *Manduca*, Sun *et al*²⁸ have demonstrated that the *Manduca* homologue of SUG1 (S8) is increased in expression at mRNA and protein levels in the ISM committed to die. Light microscopical immunocytochemistry showed that S8 was located predominantly in nuclei prior to the commitment of the ISM to die and then accumulated in cytoplasm at the time of death. This is very similar to our observations with immunogold EM where S10b moves from nuclear heterochromatic locations in the

intact fibres to areas of degeneration in disrupted fibres (Figures 2b, 3c and 4b). It seems highly likely that S8 and S10b are involved in similar (probably proteasomal) functions, possibly involving helicase activity,²⁹ to regulate degradation of critical nuclear transcription factors together with S6, S7 and 20S components at stage 0. The importance of S10b in selected nuclear functions has already been demonstrated in *S. cerevisiae*.³⁰ During muscle fibre degeneration, on the other hand, our observations suggest that S8 and S10b become involved in degradation of myofibrillar protein in the cytoplasm.

In summary, therefore we observe that certain regulatory ATPase subunits localise only in the nucleus at stage 0 (S6 and S10b), while others are only in degenerating cytoplasm at stage 7 (S6' and S10b), whereas a third category can be found in both places throughout the PCD (S7 and also the 20S core, Table 1). Proteasomes can play two leading roles in selective protein degradation. They can control the steady state concentrations of key nuclear regulatory proteins such as transcription factors in response to intracellular and extracellular signals. They can also remove bulk cytoplasmic proteins in response to physiological signals. In both cases proteins are targeted by ubiquitination. Thus in *Manduca* ISM there is a marked increase in ubiquitin conjugates when muscle cell death is induced¹⁰ and a shift in the major targets for protein degradation from nuclear proteins at stage 0 to myofibrillar protein at stage 7/8.

The six regulatory ATPases are normally believed, at least in yeast, to exist together in the 'base' complex of the 19S particle making pairwise interactions.^{13,16} Thus one would expect each ATPase to show similar subcellular distribution. However, our immunogold results, showing strikingly different distributions in ISM, are most readily interpreted in terms of several kinds of proteasomes with different subunit compositions in the degenerating ISM. The quite distinct phenotypes of yeast cells with individual mutant proteasomal ATPases suggests that each ATPase has specific non-redundant roles in proteasome function.^{30,31} This may involve recognition, recruitment or unfolding of specific substrates by different subunits of the same particle. However, an alternative in our studies may be the presence of different proteasomal complexes with distinct complements of ATPases. Thus our data could be interpreted to mean that whereas proteasomes containing S7 function in nucleus, cytoplasm and degrading fibres, complexes enriched in S6 function mainly in degradation of nuclear regulatory proteins, and those enriched in S6' mainly in cytoplasmic and fibre degradation. The movement of S10b (and S8,²⁸) from nucleus to cytoplasm and degrading fibres would reflect the shift in protein degradation from nuclear to myofibrillar targets when muscle cell death ensues. Thus proteasomes enriched in these two ATPases would be most suited to such a function. This could be simply a matter of specificity of substrate selection or processing, or it could reflect the fact that proteasomes enriched in these two ATPases can degrade substrates more rapidly than others during this period of mass protein elimination.

It seems less likely that the presence of ATPases in other, non-proteasomal complexes can fully explain our data, although the presence of large non-proteasomal complexes containing these ATPases with other functions cannot be completely ruled out.^{32,33}

Materials and Methods

Insects

Tobacco hornworms, *Manduca sexta* (L.) (Lepidoptera; Sphingidae) were reared at 25°C, under a 17 h light / 7 h dark photoperiod, on a wheat germ-based artificial diet using standard procedures.³⁴ Different stages of pharate adult development were recognised by a staging scheme adapted from that of Schwartz and Truman³⁵ and described fully by Samuels and Reynolds.³⁶ Briefly, stages of development mentioned in this paper are as follows: pharate adult stage 0: greater than 100 h before eclosion; pharate adult stage 7: about 6 h before eclosion; stage 8+15 h: 15 ± 0.5 h after eclosion.

Antibodies

Polyclonal antisera were generated against proteasome ATPase subunits (S6, S6', S7 and S10b) and the 20S core. The anti-S6 antibody was raised against recombinant protein expressed from pSMS73c.¹¹ The anti-S6' antibody was raised against a histidine-tagged human S6' expressed from a pET recombinant plasmid (Novagen, Abingdon, UK). The full open reading frame of S7 from HeLa cell cDNA was PCR amplified, ligated into pRSET (Invitrogen, San Diego, USA) and expressed in *E. coli* strain BL21(DE3) (Invitrogen, San Diego, USA). The expressed protein was purified via nickel affinity chromatography. Approximately 75–100 µg of purified protein was used for the primary immunisation and each boost over four injection sites subcutaneously. The protein was used in conjunction with incomplete Freund's adjuvant (Sigma, St. Louis, USA). Rabbits expressing high titre antibodies (checked via Western blot) were bled out after approximately 10 weeks. The S10b coding sequence¹⁴ was ligated into pRSETC (Invitrogen, San Diego, USA) and expressed in *E. coli* strain BL21(DE3) (Invitrogen, San Diego, USA). The purified histidine-tagged S10b ATPase was then used for immunisation of rabbits. For raising the 20S core antibody, the amino acid sequence of a 35 kDa subunit of the *Drosophila melanogaster* 20S proteasome³⁷ was evaluated for potential peptide antigens. The N-terminal 18 amino acid peptide (MFRNQYDSDV TVWSPQGR) was chosen in a hydrophobicity analysis of the sequence to use as the antigen. It was not only hydrophilic but appeared to be well conserved (it differs by only 1 amino acid from the corresponding rat sequence and by 3 amino acids from the rice orthologue). The peptide was chemically synthesised, covalently coupled to thyroglobulin with glutaraldehyde, dialysed and sterile filtered. The coupled peptide was then used for immunisation of rabbits in combination with Imject Alum adjuvant (Pierce, Rockford, USA). Using five boosts, rabbits expressing good titres of antibody (tested via ELISA) were bled out after 8 weeks. Antisera were affinity purified by binding to antigens coupled to an N-hydroxysuccinimide (NHS)-activated Sepharose column (Hi-Trap, Pharmacia, Uppsala, Sweden) and eluted with 0.2 M glycine, pH 2.5.

Gel electrophoresis and immunoblotting

Abdominal ISMs of individual staged *Manduca* pupae were rapidly dissected free in saline and all adhering fat body and trachea removed.

The samples were homogenised in ice cold 20 mM Tris-HCl, 50 mM NaCl, pH 7.5 and centrifuged at $9600 \times g$ in a fixed angle rotor for 5 min. The supernatant was mixed with loading buffer and boiled for 5 min, 10 μ g of total protein was subjected to electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gels.³⁸ Proteins were transferred onto nitrocellulose (Hybond Super-C, Amersham, Little Chalfont, UK) for 1 h at 100 V according to the procedure of Towbin et al.³⁹ Following transfer, membranes were blocked in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) containing 5% non-fat dried milk for 1 h, at 20°C. The blocked membranes were then incubated in the presence of affinity purified rabbit anti-ATPase subunit (S6, S6', S7 and S10b) antibodies in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween20, pH 7.5) containing 3% nonfat dried milk overnight at 4°C. Antibody binding was visualised by BCIP/NBT liquid substrate system (Sigma, St. Louis, USA) using anti-rabbit secondary antibodies labelled with alkaline phosphatase.

Immunogold electron microscopy

Muscle samples of pupal and newly emerged adult stages were dissected and fixed in 2% (w/v) paraformaldehyde/0.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1% (w/v) sucrose and 2 mM CaCl_2 for 1 h at room temperature. Samples were washed in 0.1 M cacodylate buffer (pH 7.4) and embedded in Durcupan (Fluka, Buchs, Switzerland). Immunogold labelling was carried out on ultrathin sections by a post-embedding biotin-antibiotin bridge method. The first, rabbit anti-proteasome subunit antibodies were used at a dilution of 1:100 followed by a biotinylated goat antibody to rabbit IgG (Vector Labs., Peterborough, UK; 1:200 dilution) and finally an anti-biotin gold (10 or 20 nm, Bio-Cell, Cardiff, UK; 1:100 dilution). Immunostained sections were counterstained with uranyl acetate and lead citrate and examined in a JEM-100CX II electron microscope (Jeol, Tokyo, Japan) operating at 60 kV.

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