Expression of a 26S proteasome ATPase subunit, MS73, in muscles that undergo developmentally programmed cell death, and its control by ecdysteroid hormones in the insect *Manduca sexta*

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Abstract MS73, an ATPase regulatory subunit of the 26S proteasome in the moth *Manduca sexta*, is shown to be expressed at a high level only in muscles that are undergoing developmentally programmed cell death, or which are destined to do so. The amount of MS73 is increased by more than two-fold just before death in each of three different muscles that die at different times, under different developmental controls. An ecdysteroid (moulting hormone) agonist, RH-5849, that prevents the occurrence of programmed cell death in two of these muscles also prevents the normally occurring rise in level of MS73 in these muscles. This evidence is consistent with a role for MS73 in programmed cell death.

Key words: Proteasome; ATPase; Programmed cell death; *Manduca sexta*; Ecdysteroid agonist; RH-5849

1. Introduction

Evidence is now mounting that intracellular proteolysis is involved in programmed cell death [1]. In this paper we present evidence that a regulatory ATPase subunit of the 26S proteasome may have a role in the programmed death of insect muscle during metamorphosis.

The ventral intersegmental muscles of abdominal segments 3–6 (ISM3–6) of the tobacco hornworm moth, *Manduca sexta*, which are retained from the larval condition through the 3-week pupal stage, are used during the emergence (eclosion) of the adult moth from the pupal case, but then degenerate and die. The muscles begin to regress in the ca. 48 h period prior to eclosion, a change dependent on the pre-emergence decline in titre of the insect's ecdysteroid moulting hormone, 20-hydroxyecdysone, and then abruptly regress during the ca. 36 h following eclosion [2]. These muscles have recently been extensively investigated as a model of developmentally programmed cell death (e.g. [3–7]), although the manner in which this death occurs can be distinguished from the typical apoptosis of mammalian cells in a number of ways [8].

We have previously shown [3] that MS73, a 47 kDa ATPase subunit of the 26S proteasome, is expressed in these muscles in a way that suggests an association with the developmentally programmed degeneration and death of these cells. The evidence for this is as follows:

A cDNA clone encoding MS73 was isolated from a library made from ISM3–6 mRNA at stage 7 of pharate (pre-emergence) adult development. This is a stage at which the ISM are already regressing, and are preparing to die when the moth ecloses. MS73 mRNA was found to be present in pharate adult ISM3–6 at a low but detectable level prior to stage 4 (about 68 h before eclosion) when there was an abrupt increase in expression. Western blotting showed that the amount of MS73 protein increased from stage 4 onwards, notably at stages 7 (about 6 h before eclosion) and 8 (time of eclosion). MS73 was identified as a part of the 26S proteasome complex by its inferred amino acid sequence, which shows homology to other proteasome ATPases, and also by its presence in a very high molecular weight fraction isolated from ISM3–6 by glycerol gradient centrifugation. This fraction possessed the multiple proteinase activities expected of the 26S proteasome, and could be distinguished from the smaller 20S proteasome which lacks the regulatory 19S 'caps' [9].

MS73 is only one of a number of 26S proteasome subunits [9]. We have recently shown [10] that in addition to MS73, at least three other presumed regulatory 26S proteasome subunits with ATPase domains, and one non-ATPase subunit, also appear at higher levels in ISM3–6 just before the time of eclosion than at earlier stages. Moreover, Jones et al. [5] have independently shown that changes also occur in the proteasome's multicatalytic proteinase core (20S proteasome) in this tissue at the same time.

The possible role of 26S proteasome regulatory subunits in the programmed cell death of insect muscle is of considerable interest, since it has recently been shown that specific inhibitors of proteasome proteinase activity can prevent the death of thymocytes exposed to inducers of apoptosis [11], and NGF-deprived sympathetic neurons [12] implying that proteasome activation may also play an important role in 'typical' apoptotic events.

To investigate further the possible association between MS73 and programmed cell death, we have studied the occurrence of MS73 in other tissues of the insect, including muscles that undergo programmed cell death at different times, and how these levels of MS73 change during development. We have also examined the effect of hormonal treatments on MS73 expression and programmed cell death.

2. Materials and methods

2.1. 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 [13]. Different stages of pharate adult development were recognised by a staging scheme adapted from that of Schwartz and Truman [2] and described fully in [14]. Briefly, stages of development mentioned in this paper are as follows: L5d4:feeding stage of the fifth instar, where

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the first day after ecdysis from the fourth stage is designated d0; W0: the 24 h period following the occurrence of wandering (when the insect ceases to feed); W1 (etc.): the days following W0; P0: the 24 h period following pupal ecdysis; P1 (etc.): the days following P0; pharate adult stage 0: greater than 100 h before eclosion; pharate adult stage 3: about 80 h before eclosion; pharate adult stage 7: about 6 h before eclosion; stage 8: eclosion ±0.5 h; stage 8+15 h: 15±0.5 h after eclosion.

2.2. Experimental treatments and tissue sampling

The ecdysteroid agonist RH-5849 (kindly given by Dr G. Carlson, Rohm and Haas Co., Spring House, PA, USA) was injected (10 μg) in 10 μl of ethanol into the lateral dorsal thorax of the insect using a Hamilton syringe with a fixed 28 swg needle. Control insects received 10 μl ethanol alone. Tissue samples taken from insects were dissected under a simple insect saline solution [15], immediately frozen on a metal surface cooled with liquid nitrogen, and kept at −70°C until needed.

Tissue samples were ground in a glass-glass homogeniser in TBS (20 mM TRIS, 0.05 M NaCl, pH 7.5) and briefly centrifuged (3 min, 12 500×*g*). The protein content of the supernatant was determined by the Bradford dye-binding method [16] using a BIO-RAD kit.

2.3. Antibodies

Polyclonal antiserum was generated against an N-terminal fusion protein of MS73 [3]. It was affinity purified by adsorption to N-terminal fusion protein linked to an *N*-hydroxysuccinimide (NHS)-activated Sepharose column (Pharmacia Hi-Trap).

2.4. Polyacrylamide gel electrophoresis and immunoblotting

Tissue extracts were dissolved in Laemmli buffer and boiled for 5 min. Polypeptides were separated on 10% SDS-polyacrylamide gels as described by Laemmli [17] using a Mini-PROTEAN II apparatus (BIO-RAD). An amount of extract equivalent to 10 μg total protein was loaded in each lane. After electrophoresis the separated polypeptides were transferred electrophoretically onto Hybond-C super nitrocellulose membranes (0.45 μm, Amersham) and developed according to Towbin et al. [18].

The immunodetection of MS73 proteasome subunits was carried out using the affinity purified antibodies described above. Primary antibodies were visualised with a secondary anti-rabbit antibody labelled with alkaline phosphatase (Sigma) for development with the BCIP/NBT liquid substrate system (Sigma).

2.5. Analysis of immunoblots

Digital images of the stained immunoblots were acquired using an Epson desk scanner and a Macintosh computer, and manipulated using Adobe Photoshop. Individual bands were quantified by multiplying area×mean density. We verified in a separate experiment (not shown) that this measure of band intensity varied in a linear fashion with varying amounts of protein. The values obtained were used to calculate changes in the levels of MS73 relative to stage 8+15 h ISM3–6 which was arbitrarily assigned as 100%.

3. Results

3.1. Tissue distribution of MS73

An affinity purified antibody directed against the N-terminal MS73 fusion protein labelled only a single sharp band at 47 kDa in immunoblots of TBS-soluble protein from a range of tissues (Fig. 1). MS73 was present at high level only in extracts of intersegmental muscle (ISM3–6). Since an equal amount of total protein was loaded in each lane, band intensity, estimated from the product of area and mean density of staining, is taken to indicate the relative level of MS73 in each tissue.

As previously observed [3], MS73 levels in ISM3–6 changed dramatically during pre-emergence adult development, being present at high level only during the later stages of preparation for eclosion and after this event. Levels of the protein did not change appreciably with developmental stage in the other

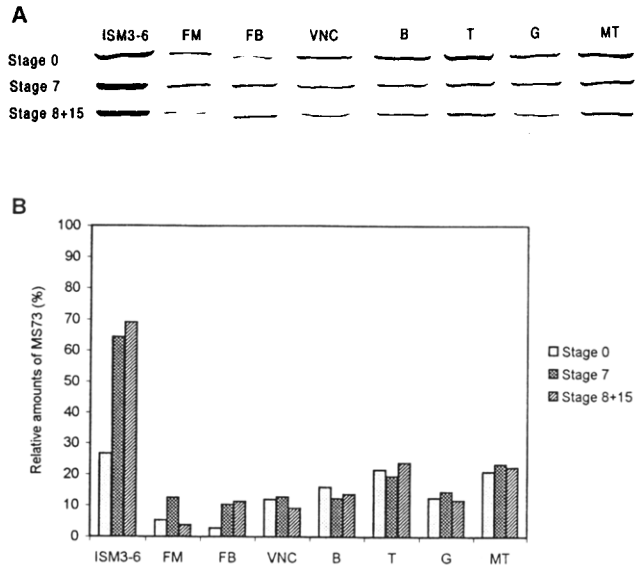

Fig. 1. MS73 protein in a range of tissues in pharate adult *Manduca sexta*. A: Immunoblots of SDS-polyacrylamide gels. 10 μg of total TBS-soluble protein was loaded in each well. B: Quantitative estimates of the amounts of MS73 in each of the tissues obtained from digital images of the bands shown in A. The stages shown are stage 0, about 100 h before adult emergence; stage 7, about 6 h before adult emergence; stage 8+15 h, about 15 h after emergence. ISM3–6 regresses during stage 7 and dies in the 36 h following eclosion. (ISM3–6: abdominal intersegmental muscle segments 3–6; FM: flight muscle; FB: fat body; VNC: ventral nerve cord; B: brain; T: testis; G: midgut; MT:- Malpighian tubules).

tissues examined. Fig. 1 shows that MS73 was present at similar low levels in most of the tissues examined (flight muscle, fat body, ventral nerve cord, brain and midgut) at pharate adult stages 0 and 7 and also in adult insects that had emerged 15 h previously (stage 8+15 h). The levels of MS73 were somewhat higher in testis (T) and Malpighian tubules (MT, excretory organs), but these were much lower than in ISM3–6, and did not change with development in the range of stages examined. The level of MS73 in ISM3–6 was 2.4× higher at stage 7 than at stage 0. The level of MS73 increased only slightly during the period between stage 7 and stage 8+15 h.

3.2. MS73 and intersegmental muscle cell death in pre-adult development

As noted by Schwartz et al. [19], the ventral abdominal intersegmental muscles do not all undergo programmed cell death at the same time. While the muscles in abdominal segments 3–6 (ISM3–6) regress just before adult ecdysis, and then degenerate immediately afterwards, the muscles of abdominal segments 1 and 2 (ISM1–2), 7 and 8 (ISM7–8), have already disappeared long before this time. In these muscles, programmed cell death is associated with the metamorphic transition from larva to pupa, occurring just after pupal ecdysis.

We first established that ISM1–2 and ISM7–8 were present at the time of pupal ecdysis (stage P0), but then degenerated rapidly, so that by 48 h after ecdysis (stage P2) only very thin and fragile fibres remained. By 72 h after ecdysis the muscles could no longer be seen. By contrast, ISM3–6 remained unchanged in appearance during this period.

MS73 levels were estimated from immunoblots of TBS-soluble protein from ISM1–2, ISM3–6, and ISM7–8 in insects undergoing development from feeding fifth stage larva to

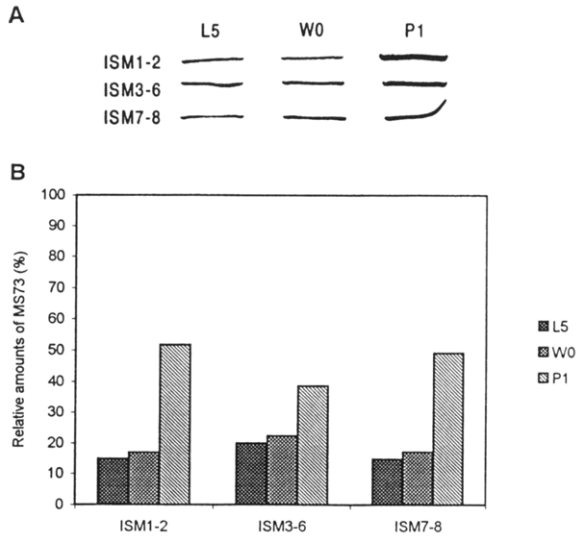

Fig. 2. MS73 protein in different regions of the abdominal intersegmental muscles at different stages of development. A: Immunoblots of SDS-polyacrylamide gels. 10 μg of total TBS-soluble protein was loaded in each well. B: Quantitative estimates of the amounts of MS73 in each of the tissues obtained from digital images of the bands shown in A. ISM1–2 is the intersegmental muscle from abdominal segments 1 and 2, and so on. The stages shown are L5, fifth stage feeding larva about 8 g in weight, about 12 h before wandering; W0, fifth stage larva 0–24 h after wandering; P1, pupa 24–48 h after ecdysis. ISM1–2 and ISM 7–8 are dying at stage P1. ISM3–6 survives until adult eclosion, some 21 days later.

emerged pupa. Fig. 2 shows that the amounts of MS73 protein in all three muscles of feeding larvae (L5) are similar to those in newly wandered larvae (W0, a stage when the larvae cease to feed and prepare to pupate). In newly emerged pupae (P1, 5 days after wandering), however, all the intersegmental muscles show much higher levels of MS73. The increase was most marked in the muscles which die at this time, ISM1–2 and ISM7–8 (increases of 2.8× and 2.7× respectively), so that in these muscles the intensity of the MS73 bands was comparable to that seen in ISM3–6 of pharate adult moths just before and after emergence (Fig. 1). ISM3–6, however, which does not undergo programmed cell death at this time, also showed a clear increase (1.8×) in the level of MS73, although not to the levels seen in muscles that die (ISM1–2, 7–8).

3.3. MS73 and cell death in larval proleg muscles

Programmed cell death also occurs during metamorphosis in other lepidopteran muscles. The degeneration and death of the larval proleg retractor (plantar retractor) muscles (PRM) have been previously studied [20] in the wax moth, *Galleria mellonella*, in which the death of these muscles occurs after pupal ecdysis. In *Manduca*, however, we have found that the muscles die rather earlier than in *Galleria*, disappearing during the period between wandering and pupal ecdysis (Löw and Reynolds, unpublished). In *Manduca* the PRM persisted with essentially unaltered appearance until stage W2 (2 days after the cessation of feeding), but then degenerated rapidly, being represented only in the form of thin, fragile fibres 1 day later at stage W3. This change coincided with the start of deposition of the new pupal cuticle underneath the old larval cuticle, and the loss of mobility of the larval prolegs.

Immunoblots of TBS-soluble protein from the PRM (Fig. 3) showed that MS73 was present at a relatively low level at stage W0, before signs of programmed cell death were evident, but that the amount of MS73 in the muscle increased markedly on day W1 and reached a still higher level on day W2. Samples could not be taken at stage W3 because the muscles were too delicate to collect. The increase in MS73 from stage W0 to stage W2 was 2.7×. The final level of MS73 band intensity was comparable to that seen in ISM3–6 at the time of adult eclosion.

3.4. Hormonal manipulation of MS73 levels

Schwartz and Truman have shown that in *Manduca*, both the pre-eclosion regression and the rapid post-eclosion death of the adult insect's ISM3–6 are under the control of the ecdysteroid moulting hormone, 20-hydroxyecdysone (20HE) [2]. Programmed cell death of these muscles can occur only following the normal pre-eclosion decline in titre of this hormone. The administration of exogenous 20HE delays or prevents completely both these processes. Injections of 20HE are much less effective than continuous infusion, however, presumably because the hormone is rapidly degraded in vivo.

We therefore used a synthetic, non-metabolisable non-steroidal ecdysteroid agonist, RH-5849 [21,22], to test whether MS73 expression is regulated by ecdysteroid action in the same way as is cell death. We found that a single injection of 10 μg RH-5849 given to stage 3 pharate adult insects completely prevented both adult eclosion and the degeneration of ISM3–6. This treatment also prevented the normally occurring increase in level of MS73 in ISM3–6 (Fig. 4A), supporting the hypothesis that this proteasome subunit is in some way involved with programmed cell death.

Injection of RH-5849 (10 μg) into earlier stages of *Manduca* had different effects on programmed muscle cell death according to the stage that was injected and the muscles concerned. When the ecdysteroid agonist was given to feeding larvae just prior to wandering, the increase in MS73 level normally seen in PRM 2 days later was completely prevented, as was pro-

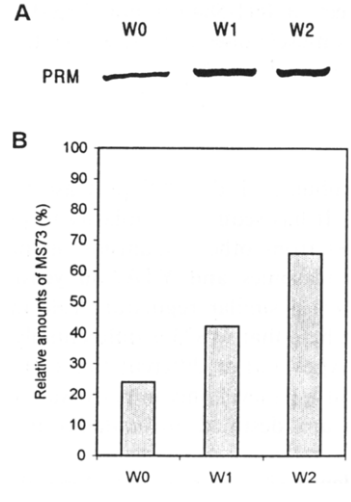

Fig. 3. MS73 protein in proleg retractor muscle (PRM). A: Immunoblots of SDS-polyacrylamide gels. 10 μg of total TBS-soluble protein was loaded in each well. B: Quantitative estimates of the amounts of MS73 in each of the tissues obtained from digital images of the bands shown in A. The stages shown are W0, W1 and W2, which are fifth stage larvae 0–24, 24–48 and 48–72 h after wandering respectively.

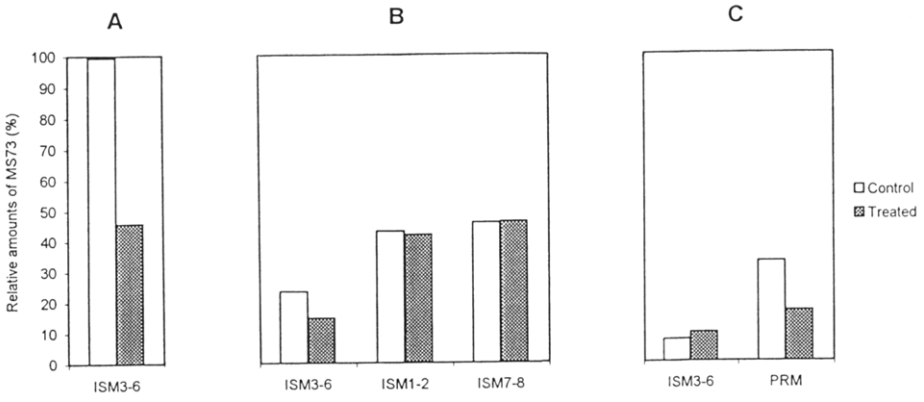

Fig. 4. Effect of RH-5849 on MS73 protein levels in three different muscles. A: ISM3–6. RH-5849 (10 μg) was given at stage 3. This treatment arrested pharate adult development and prevented eclosion. Samples were taken when controls (injected with vehicle) had reached stage 8. B: ISM1–2 and ISM7–8. RH-5849 (10 μg) was given at stage W2. This treatment prevented pupal ecdysis. Samples were taken after 72 h when controls had successfully shed the larval cuticle (i.e. were P0). C: PRM. RH-5849 (10 μg) was given to day 4 fifth instar feeding larvae (i.e. on the day before wandering). This treatment did not prevent wandering. Samples were taken after 24 h, i.e. on day W0.

grammed death of that muscle (Fig. 4C). By contrast, the effect of RH-5849 on ISM1–2 and 7–8 was quite different. As shown above, these muscles normally degenerate during the 72 h following pupal ecdysis, expressing MS73 at a high level on day P0. When RH-5849 (10 μg) was given on day W2, the treatment prevented the occurrence of pupal ecdysis, as would be expected from the similar effect of 20HE [2]. Despite this, the hormone agonist had no effect on either the programmed death of ISM1–2 and ISM 7–8 (which took place on time, as though pupal ecdysis had taken place) nor on the level of MS73 in these muscles, measured after 3 days when the controls had reached stage P0 (Fig. 4B). This suggests that in the case of ISM1–2 and ISM 7–8, programmed cell death is not dependent on a normally occurring decline in ecdysteroid titre. All the experimental results are however consistent with a role for MS73 in the cell death process. Interestingly, however, the treated insects did show a reduction in the level of MS73 present in ISM3–6 (Fig. 4B). Clearly this effect of RH-5849 is not directly related to cell death, since this muscle does not die at this time in either the controls or the treated insects.

4. Discussion

MS73 is a subunit of the 26S proteasome in the insect *Manduca sexta*. It has sequence similarity to proteasome regulatory ATPases from other organisms, in particular S6 in mammalian proteasomes and YTA2 in yeast, and may be presumed to have a similar regulatory role in *Manduca* [3]. We have shown here that MS73 is differentially expressed in a variety of cell types and at different times. It is evident that MS73 is found at high level only in body wall muscles that are undergoing or are destined to undergo programmed cell death.

MS73 was identified from a cDNA clone, the library being obtained from late pharate adult abdominal intersegmental muscle (ISM3–6), a tissue which degenerates around this time. The mRNA from which the library was constructed was taken from ISM3–6 at a time when it was about to undergo programmed death. MS73 immunoreactivity has now been found to be present in two other muscle types that undergo developmentally programmed death, the intersegmental muscles ISM1–2 and ISM 7–8, which die just after pupal ecdysis, and the proleg retractor muscle (PRM), which dies just before pupal ecdysis. In all three muscle types, the level of MS73 more than doubles just before death occurs. The amount of MS73 in these muscles at this time, relative to total TBS-soluble protein, was with a single exception more than twice that seen in any other tissue at any time. This suggests that MS73 may be involved in the cellular processes that lead to programmed death. The single exception will be discussed further below.

Treatment of insects with RH-5849, an ecdysteroid agonist, had different effects on different muscles, presumably according to the mechanism whereby programmed muscle cell death is regulated. In the case of ISM3–6 and PRM, treatment with RH-5849 prevented both cell death and the accompanying rise in MS73 level. Death of ISM3–6 is known [2] to be dependent on the decline in ecdysteroid titre which normally occurs before adult eclosion, and the repression of both cell death and MS73 expression in this muscle by RH-5849 is in accord with this. The similar effect of RH-5849 on PRM suggests that in this muscle too, programmed death is dependent on a falling ecdysteroid titre. By contrast, RH-5849 failed to prevent cell death in ISM1–2 and ISM7–8, and also did not prevent the associated rise in MS73 level. This implies that in this muscle cell type, death is not regulated by the falling ecdysteroid titre, and that in these cells MS73 expression is regulated by other factors.

The single exception to the general pattern of results discussed above is that in which MS73 levels were found to be high in ISM3–6 just after pupal ecdysis (although not as high as in dying muscles). This is a problem for the hypothesis that MS73 plays an obligatory and causal role in programmed muscle cell death. This muscle type undergoes programmed death, and does so at a time (adult eclosion) when MS73 levels are high, but the earlier post-pupal ecdysis rise in MS73 level does not lead to the death of ISM3–6 at this time. Thus if MS73 is indeed involved in the machinery of programmed cell death, elevated expression of MS73 alone is not sufficient to ensure that death ensues. It could be that the lack of some other factor, perhaps another regulatory protea-

some subunit, that prevents death. Alternatively, it is possible that the level of MS73 needs to pass some critical threshold which is not exceeded in ISM3–6 at stage P1.

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