The S10b (SUG2) ATPase cDNA has been cloned by reverse transcription-polymerase chain reaction/rapid amplification of cDNA ends from mRNA of intersegmental muscles of the tobacco horn moth (*Manduca sexta*). The S10b ATPase is a component of the 26 S proteasome, and its concentration and that of its mRNA increase dramatically during development in a manner similar to other ATPases of the 19 S regulator of the 26 S proteasome. The S10b and S6' (TBP1) ATPases are also present in a complex of ~220 kDa in intersegmental muscles. The 220-kDa complex markedly activates (2–10-fold) the 26 S proteasome, even when bound to anti-S10b antibodies immobilized on Sepharose, and increases in concentration ~5-fold like the 26 S proteasome in the intersegmental muscles in preparation for the programmed death of the muscle cells. A similar activator complex is present in human brain and placenta. Free activator complexes cross-activate: the *Manduca* complex activates rat skeletal muscle 26 S proteasomes, and the placent complex activates *Manduca* 26 S proteasomes. The placental activator complex contains S10b and S6', but not p27. This 220-kDa activator complex has been evolutionarily conserved between species from insect to man and may have a fundamental role in proteasome regulation.

Type II programmed neuromuscular cell death is a feature of some abdominal motor neurons and intersegmental muscles (ISM)1 at eclosion in the tobacco horn moth (*Manduca sexta*) (2–4). After emergence, these cells die within 24–36 h in response to changes in circulating levels of ecdysteroid hormone. Previous studies have shown that eclosion is preceded in ISM by a massive increase in polyubiquitin gene expression (5) and a large increase in ubiquitinylated proteins (6) and in the levels of proteasomes (7, 8). In the muscles of larvae at stage 0, before the hormone-dependent changes in gene expression activate the death process, proteasomes appear to be depleted in some regulatory ATPases (e.g. S4 and S7).5 Marked increases in several ATPases (S4, S6, and S7) then occur, so that by stage 8, just before cell death, the 26 S proteasomes prepared from muscle contain at least four of the regulatory ATPases (S4, S6, S6', and S7). The simplest explanation is that proteasomes are increased in number and equipped with the ATPases needed to degrade the accumulating multi-ubiquitinylated proteins during the programmed elimination of the muscles (8). The first observations that the ubiquitin/26 S proteasome system is involved in programmed cell death in *M. sexta* (5–8) have been followed by other data that implicate proteasomes in the decisions that favor either cell death or survival (9–16).

There are six ATPase molecules found in the 19 S regulator of the 26 S proteasome (1). As an extension of studies on these ATPases in programmed cell death in ISM during eclosion in *M. sexta* (8), we have cloned a second S10b (SUG2) ATPase and shown that this ATPase is not only associated with 26 S proteasomes, but is also found in a much smaller complex, of ~220 kDa, with the S6' (TBP1) ATPase. The concentration of the 220-kDa complex increases in ISM during programmed cell death at the same time as that of the 26 S proteasome, suggesting a role for the 220-kDa complex in muscle cell death. A modulator complex containing the S10b and S6' ATPases and a p27 protein has been previously described (17). Complexes similar to the *Manduca* 220-kDa complex are present in human brain and placenta and contain the S10b and S6' ATPases, but not p27. The 220-kDa complexes can activate preparations of 26 S proteasomes across species barriers, indicating the evolutionary conservation of these proteasomal activators. Their potential roles in the proteolytic mechanisms of the 26 S proteasome and other possible functions of the S10b, S6', and other ATPases in the cell are discussed.

**EXPERIMENTAL PROCEDURES**

**Insect Culture and Staging**—The insects were raised, the stages of pre-ecdysial development recognized, and muscles collected as described previously (8).

**Reverse Transcription-PCR Amplification of M. sexta S10b (SUG2) cDNA**—Degenerate primers matching the ATPase boxes A and B (18) were used to clone several different ATPase sequences from *Manduca* genomic DNA. The two primers TAYGGNCCNCCNGGNACNGGNA (corresponding to the protein sequence YGPPGTTG) and GGNGCRT-
FIG. 1. cDNA and protein sequences of *M. sexta* S10b ATPase. Potentially important motifs within the protein sequence are underlined; these are as follows: residues 33–57, a putative coiled-coil motif (amino acids on the hydrophobic face are underlined); residues 180–188, Walker nucleotide-binding motif A; residues 238–244, Walker nucleotide-binding motif A; residues 284–286 and 297–304, putative DNA helicase motifs (40, 41).

CRATYTCRTCTRATRAA (antisense strand for the protein sequence FI-DEIDA) were used at a concentration of 0.5 μM in a 50-μl PCR containing 50 μl dNTPs, 200 ng of genomic DNA, and 2.5 units of Taq DNA polymerase (Roche Molecular Biochemicals, Lewes, United Kingdom). A 200-base pair product was cloned into the polymerase (Roche Molecular Biochemicals, Lewes, United Kingdom). (40, 41).

304, putative DNA helicase motifs motif B; and residues 284–286 and 297–304, putative DNA helicase motifs (40, 41).

...after concentration, the 3′ end of a clone that showed good homology to other proteasomal ATPases, but appeared to represent a distinct gene (“AAA (ATPases associated with diverse cellular activities) clone”). RNA (1 μg) obtained from stage 7 *Manduca* ISM (19) was reverse-transcribed (37 °C, 2 h) in the following 20-μl reaction: 100 ng of an oligo(dT) primer. Excess primers were removed using 20 units of terminal deoxynucleotidyltransferase (Life Technologies, Inc.). Next, 2 μl of the reaction was used in a 50-μl reverse-transcription reaction: 100 ng of genomic DNA, and 2.5 units of the proofreading DNA polymerase (Stratagene), 500 μM dNTPs, and 25 pmol of primers ATGCCTGCCGGACCTTCC and another AAA clone-specific primer (CAGACTATTATTAATTACAGAT). A 1350-base pair PCR product was ligated into the EcoRV site of the pSK plasmid and sequenced.

Finally, the full-length coding sequence of the *Manduca* homologue of S10b was amplified (annealing temperature of 55 °C, 35 cycles) with 2.5 units of the proofreading *Pfu* DNA polymerase (Stratagene), 500 μl dNTPs, and 25 pmol of primers ATGCCTGCCGGACCTTCC and another AAA clone-specific primer (AAATCTATGAAGTATTACAGAT). A 1350-base pair PCR product was ligated into the EcoRV site of the pSK plasmid and sequenced.

Preparation of Tissue Extracts—Frozen (−70 °C) ISM (500–1000 mg) taken from different developmental stages of *M. sexta* was homogenized in 4 volumes of homogenization buffer (20 mM Tris-HCl, 2 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol, and 10% glycerol (pH 7.5)) using a Polytron homogenizer. The homogenate was centrifuged twice at 10,000 × g for 10 min at 4 °C, and the supernatant was collected (S2, soluble muscle extract). Human placental extract was prepared as described for ISM. Homogenized human brain was similarly processed (17, 18) and named pSK.S10b.cds.

...in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.125 mM dNTPs, and 200 ng of genomic DNA, and 2.5 units of *Pfu* DNA polymerase (Stratagene), 500 μM dNTPs, and 25 pmol of primers ATGCCTGCCGGACCTTCC and another AAA clone-specific primer (AAATCTATGAAGTATTACAGAT). A 1350-base pair PCR product was ligated into the EcoRV site of the pSK plasmid and sequenced.

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Protein and chymotrypsin activity were determined and immunoassay fractionated by centrifugation through a 10–40% glycerol gradient. Assayed for peptidase activity (insect muscle, 5 soluble proteins were fractionated in the same way. Fractions were applied to a Superose 12 column (Amersham Pharmacia Biotech) at room temperature (8). Eluted protein was dialyzed against 10 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100 overnight at 4 °C, freeze-dried, checked for purity by SDS-polyacrylamide gel electrophoresis, and then used for immunization of New Zealand White rabbits. Antiserum to S10b ATPase was tested against the expressed fusion protein by Western analysis of whole cell extract from isopropyl-β-D-thiogalactopyranoside-treated E. coli BL21(DE3) cells transformed with pSK.S10b.cds.

The anti-S6 antibody was raised against the recombinant Manduca protein expressed from pSMST73e (8). Anti-S6- and anti-S10b antibodies were raised against His-tagged human S6' and S10b expressed from pET recombinant plasmids (Novagen, Abingdon, UK) as described above. Antiserum to Manduca and human S10b and human S6' were affinity-purified by binding to purified His-tagged recombinant antigen protein, coupling to CNBr-activated Sepharose (Sigma, Poole, UK), and eluting with 1 M glycine (pH 2.5). Antiserum to a control (irrelevant) peptide (AQ63, a fragment of pro-islet amyloid precursor protein) was similarly affinity-purified on immobilized AQ63 peptide.

Monoclonal antibodies to human S10b, S6', S7, and S8 ATPases and the 20 S subunits HC2/MCP20 and Z/MCP205 were kindly provided by Dr. Klavs Hendil (August Krogh Institute, University of Copenhagen). Polyclonal antiserum to human recombinant p27 was from Prof. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo, Japan).

Western Analyses—Western analyses were performed as described (8).

Immobilization of Proteasomal Activators on Anti-S10b Immunofluorescence Matrix—Affinity-purified Manduca anti-S10b and human anti-S10b (10 ml) and anti-AQ63 (equivalent to 2.5 ml of antiserum) antibodies were coupled to CNBr-activated Sepharose 4B (1 ml). Activator complexes from glycerol gradient fractions 3–5 were bound to the matrix.
Fraction no

Fig. 4. Fractions containing the 220-kDa complex are able to activate the 26 S proteasome. Different volumes of the stage 7 glycerol gradient fractions 1–8 from Fig. 2 were added to 0.5 ml of similarly prepared peak 26 S proteasome fraction, and chymotrypsin activity was assayed (see "Experimental Procedures"). Activation represents the chymotrypsin activity measured with fractions 1–8 plus 26 S proteasomes minus the sum of activity measured when fractions 1–8 and proteasomes were assayed separately. The activity of unstimulated 26 S proteasomes assayed in the same way was 450 units. Activation and proteasomes were assayed separately. The activity of unstimulated S proteasomes minus the sum of activity measured when fractions 1–8 represents the chymotrypsin activity measured with fractions 1–8 plus 26 S (rat) loading buffer (21).

Activation by inversion for 1–2 h at room temperature. After repeated washing with homogenization buffer, activator complexes were eluted from the immunoaffinity matrices for Western analyses with 1 M glycine (pH 2.5), and residual bound material was released by boiling in urea/SDS loading buffer (21).

RESULTS

Reverse Transcription-PCR/RACE of the S10b cDNA and Sequence Comparisons—As part of the characterization of all proteasomal regulatory ATPases from Manduca, multiple DNA sequences encoding the region between the two elements of the Walker ATP-binding motifs were amplified and cloned. Sequencing of individual clones revealed five different sequences, some of which bore very close homology to previously identified proteasomal ATPases. One clone that bore less homology to known proteasomal ATPases was chosen for further study, and full-length cDNA sequences were obtained using PCR/RACE. The DNA and predicted protein sequences of the ATPase shown in Fig. 1 are closely homologous to the 19 S regulatory S10b (SUG2) ATPase (1).3 As is the case for many other members of this subgroup of the AAA superfamily, the S10b ATPase has a putative coiled-coil motif, the Walker A and B nucleotide-binding motifs, and putative DNA helicase motifs. The S10b ATPase mRNA and protein increase ~5-fold in concentration in the muscles in preparation for cell death (data not shown). Again, these changes are similar to those observed for Manduca S4, S6, and S7 ATPases during the same period (8). The S10b ATPase is a component of the 26 S proteasome as shown by glycerol gradient centrifugation (Fig. 2); this analysis also demonstrates that the increase in the tissue content of the S10b ATPase is mainly associated with the 26 S proteasome.

The S10b ATPase Is Present in the Cell Independent of the 26 S Proteasome—Overexposure of the enhanced chemiluminescence signals in the Western analysis of the glycerol gradient fractionation of stage 7 ISM (Fig. 2B) shows that the S10b ATPase is not only present in the 26 S proteasome, but is also present in fractions (3–6) containing lower molecular mass complexes (Fig. 2C). The nature of these complexes was further investigated by gel filtration chromatography on Superose 12. Western analysis of the fractions after gel filtration shows that both the S10b and S6 ATPases elute as if present in complexes of ~220 kDa (Fig. 3, A, B, and D). The relatively weak immunoreactivity of S6 is probably due to the fact that the affinity-purified anti-S6 antibody was raised against the human S6 protein. The S6 ATPase is not enriched in complexes of this size, with the antigen detected in this region probably representing the trailing edge of proteasomal antigen spread by diffusion during chromatography (Fig. 3, E and F). The 220-kDa complex is found in muscles at the beginning (stage 0) and toward the end (stage 7) of the period of preparation for muscle cell death. The volumes of the stage 7 fractions analyzed by Western blotting were five times less than those of stage 0 since the total ATPase content of ISM increases ~5-fold during pupal development. The similar intensities of the S10b bands in the 220-kDa complex from stages 0 and 7 (Figs. 2 and 3) therefore demonstrate that, like the proteasomal ATPases (8), the concentration of the complexes containing the S10b ATPase increases ~5-fold in the muscle cells during preparation for cell death. The amounts of the ATPases in the 220-kDa complex do not change when soluble extracts are prepared from muscles in the absence of ATP, indicating that the complex containing the S10b ATPase is unlikely to be produced by dissociation of 26 S proteasomes on homogenization (data not shown). The fact that the S6 ATPase is not in the 220-kDa complex (Fig. 3, E and F)
also supports the notion that the complex is not produced by
dissociation of ATPases from the 26 S proteasome. A careful
analysis of the relative amounts of the S10b ATPase in the 26
S proteasome compared with the 220-kDa activator indicates
there is ~3–5% of the S10b ATPase in the activator relative to
the 26 S proteasome (data not shown).

**Fractions Containing the 220-kDa Complex Are Able to Acti-
vate the 26 S Proteasome**—Glycerol gradient fractions contain-
ing the 220-kDa complex from stage 7 (fractions 1–8) were
incubated with similarly prepared 26 S proteasomes to inves-
tigate whether the 220-kDa complex was able to directly influ-
ence the peptidase activity of the proteasome (Fig. 4). Fractions
enriched in the 220-kDa complex (fractions 3–6) cause consid-
erable activation of the chymotrypsin activity of the 26 S pro-
teasome in a concentration-dependent manner. The *Manduca*
activator was further characterized to determine whether it is
related to the modulator complex isolated from human erythr-
ocytes, which stimulates assembly of 26 S proteasomes from
20 S cores and 19 S regulators (17, 22, 23). These experiments
are summarized in Table I.

The capacity of the *Manduca* activator complex to increase
the chymotrypsin-like activity of fractions enriched in the 26 S
proteasome varied between different preparations, from >10-
fold (Fig. 4) to ~2-fold (Table I). This presumably reflects
variation both in the activator complex itself and in the capac-
ity of the 26 S proteasome to respond in different experiments.
Activation capacity does not appear to depend on the propor-
tions of 20 S and singly or doubly capped 26 S proteasomes in
the preparation since the glycerol gradient fractions showing
peak peptidase activity were activated by the 220-kDa complex
to a greater extent than leading or trailing edges of the protea-
some peak (Table I, Experiment B). If the activator was simply
stimulating the assembly of doubly capped 26 S complexes from
19 S and 20 S components (22, 23), we would expect the extent
of activation to be greatest on the lighter (trailing) side and
least on the heavier (leading) side of the peak. The *Manduca*
activator increases 26 S proteasome activity only in the pres-
ence of ATP (Table I, Experiment D). Indeed, in contrast to the
purified human 11 S (PA28) activator (Table I, Experiment C),
the *Manduca* 220-kDa activator did not activate purified 20 S
proteasomes (Table I, Experiments A and D), and thus is not
the *Manduca* equivalent of PA28 (24). However, the *Manduca*
220-kDa activator was able to activate a 26 S proteasome
preparation from rat skeletal muscle, demonstrating that it
can activate mammalian proteasomes efficiently (Table I, Experi-
ment D).

The data presented so far have shown that activator activity
and the S10b and S6’ ATPases copurify together. To prove that
activator activity is dependent on the S10b ATPase, activator
preparations were incubated with affinity-purified polyclonal
anti-S10b antibody bound to Sepharose beads. All of the
*Manduca* activator bound to the beads, and the immobilized
complex could still activate the *Manduca* 26 S proteasome
chymotrypsin activity (Fig. 5). The activator did not bind to
unsubstituted Sepharose. Further purification and character-
ization of the activator from *Manduca* ISM were not possible
due to the small amounts of tissue available. However, since
the activator stimulated mammalian proteasomes, human tis-
sues were analyzed to determine whether a similar human
220-kDa complex exists.

**Human Brain, Placenta, and Embryonic Kidney Cells Have
Complexes Containing the S10b and S6’ ATPases**—Glycerol
gradient fractionation of human brain cortex (Fig. 6) reveals a
distribution of 26 S proteasomes similar to insect muscle (Fig.
3). In addition, the S10b and S6’ ATPases, but not the S7 and
S8 ATPases, are found in a smaller complex (fractions 3–7)
similar to the 220-kDa complex found in insect muscle (Fig. 2).
Similar evidence for a smaller complex containing the S10b and
S6’ ATPases, but not the other ATPases, was found in early
gradient fractions prepared from other human tissues: normal
and neonatal brain and placenta and in extracts from human
embryo kidney cells (data not shown). Small complexes con-
taining the S10b and S6’ ATPases seem to be widely distrib-
uted in human cells as well as in insect muscle.

**Properties of the Placental Activator**—The human placen-
tal activator showed an activation capacity similar to that of the
*Manduca* activator in a dose-dependent manner (Table II, part
B). Interestingly, glycerol gradient fractions containing the
placental activator increase the activity of *Manduca* 26 S pro-
teasomes considerably more than placental 26 S proteasomes
(Table II, Experiments A and B). An increase in the activity of
purified human 20 S proteasomes sometimes occurs (Table II,
Experiment B, but not part C) with fractions containing the
placental activator due to the presence of the 11 S activator in
human tissues. This does not occur with the insect activator
since the 11 S activator does not appear to be present in insect
muscle (Table I). Thus, ATPase-containing activator complexes
can activate 26 S proteasomes across species barriers: *Manduca*
complexes activate rat skeletal muscle (Table I, Experi-
ment D), and placental complexes activate *Manduca* 26 S pro-
teasomes (Table II, Experiments A and B). The combined
observations indicate that the activator has been evolutionarily
conserved between species from insect to man.

The p27 Protein Subunit and Activator Complexes from Non-
red Blood Cells—A modulator complex can be purified from
bovine red blood cells (17) and contains the S10b and S6’
ATPases together with a novel p27 subunit that is claimed also
to be present in 26 S proteasomes (25). As expected, glycerol
gradient fractionation of human red blood cell fraction II (Fig.
7) confirmed that S10b, S6’, and p27 (but not the S7 ATPase)
were markedly enriched in early gradient fractions, which is consistent with earlier observations (17). However, very little S10b, S6, and p27 were present in the proteasome fractions, indicating that the high salt, ATP-depleted conditions used for isolation of fraction II (17, 20) have disrupted the proteasome and possibly the 19 S regulator. In an analogous fashion, proteasomes prepared from placental tissue frozen at $-70$ °C for several months appear to be disrupted with the vast majority of S10b and S6 in small complexes, with S7 in rather larger complexes, and with very little ATPases in particles resembling 19 S regulators or 26 S proteasomes (Fig. 8, compare with Fig. 6). However, the p27 subunit was not detected in early glycerol gradient fractions containing the S10b and S6 ATPases whether material was analyzed from frozen (Fig. 8) or fresh (data not shown) human placenta. Indeed, p27 could not be detected in placental S2 fractions (Fig. 9). More significantly, when placental activator complexes were immobilized by affinity attachment to anti-S10b antibody-Sepharose (Fig. 9), both S10b and S6 were enriched, but neither p27 or the S7 ATPase could be detected in bound material. The activator did not bind to anti-AG93 antibody-Sepharose, an irrelevant immunoaffinity control matrix, proving the specificity of the interaction with anti-S10b antibody-Sepharose (data not shown). Therefore, the p27 protein may not be widely distributed in different human tissues and does not form part of the 220-kDa activator demonstrated in this work.

**DISCUSSION**

**Proteasomes and Type II Programmed Cell Death**—Previous studies on the programmed elimination of abdominal intersegmental muscles in the tobacco horn moth (*M. sexta*) have shown that the ubiquitin/26 S proteasome system is dramatically activated to degrade muscle proteins during the death process (5–8). The combination of a massive increase in polyubiquitin gene expression, accumulation of ubiquitylated proteins, and a large increase in levels of the 26 S proteasome (8) provides the degradation machinery necessary for the proteolytic destruction of muscle proteins. The developmental changes in 26 S proteasomes occur only in the abdominal intersegmental muscles, which are destined to die, and not in flight muscles, which are needed in the emerging adult moths for locomotion (8, 26). Furthermore, at least one ATPase (S6) is expressed only at high levels in other muscles that undergo developmentally programmed cell death: increased expression of the ATPase occurs at distinct times in different muscles to coincide with the death process (27). The increase in 26 S proteasome components is hormone-regulated and suppressed by the ecdysteroid agonist RH-5849 (26, 27).

**The 220-kDa Activator and Cell Death**—In this study, we have cloned the cDNA for another *Manduca* ATPase (S10b; Fig. 1) and studied the expression pattern and biochemical properties of the protein. The S10b ATPase is a component of the muscle 26 S proteasome and shows developmental increases in expression in the intersegmental muscles (Fig. 2) similar to other ATPases studied previously (8).

The S10b ATPase is found not only in the 26 S proteasome, but also in other molecular forms in the muscle cells. Western analysis of glycerol gradient fractions shows that smaller S10b, S6', and p27 were present in the proteasome fractions, comparing with earlier observations (17). However, very little S10b, S6', and p27 were present in the proteasome fractions, indicating that the high salt, ATP-depleted conditions used for isolation of fraction II (17, 20) have disrupted the proteasome and possibly the 19 S regulator. In an analogous fashion, proteasomes prepared from placental tissue frozen at $-70$ °C for several months appear to be disrupted with the vast majority of S10b and S6 in small complexes, with S7 in rather larger complexes, and with very little ATPases in particles resembling 19 S regulators or 26 S proteasomes (Fig. 8, compare with Fig. 6). However, the p27 subunit was not detected in early glycerol gradient fractions containing the S10b and S6 ATPases whether material was analyzed from frozen (Fig. 8) or fresh (data not shown) human placenta. Indeed, p27 could not be detected in placental S2 fractions (Fig. 9). More significantly, when placental activator complexes were immobilized by affinity attachment to anti-S10b antibody-Sepharose (Fig. 9), both S10b and S6 were enriched, but neither p27 or the S7 ATPase could be detected in bound material. The activator did not bind to anti-AG93 antibody-Sepharose, an irrelevant immunoaffinity control matrix, proving the specificity of the interaction with anti-S10b antibody-Sepharose (data not shown). Therefore, the p27 protein may not be widely distributed in different human tissues and does not form part of the 220-kDa activator demonstrated in this work.

**FIG. 6.** Distribution of 26 S proteasome subunits after glycerol gradient fractionation of human brain cortex.

Glycerol gradient fractions (30 μl) from human Alzheimer’s brain cortex were analyzed by SDS-polyacrylamide gel electrophoresis and Western analysis. Transfers were incubated with hybridoma culture supernatants containing monoclonal antibodies to subunits of the 19 S complex at the following dilutions: 1:10, S6' ATPase; 1:20, S10b ATPase; 1:5, S8 ATPase; 1:10, S7 ATPase; and 1:5000 and 1:500, monoclonal antibodies MCP20 and MCP205 raised the 20 S a-subunit H2 and 20 S β-subunit Z, respectively. Proteasome chymotrypsin activity was concentrated in fractions 9–17.
kDa. The S6 ATPase is not enriched in this complex (Fig. 3, E and F). The increase in the concentration of the 220-kDa complex in the muscles from the beginning (stage 0) to the end (stage 7) of preparation for muscle death is similar to that for the ATPases of the 26 S proteasome (Figs. 2 and 3) (8). The 220-kDa complex is able to activate *Manduca* 26 S proteasomes 2–10-fold in an S10b-dependent manner (Figs. 4 and 5 and Table I).

A modulator complex containing both the S10b and S6' ATPases plus the p27 protein has been purified from bovine red cells and characterized as an activator of the 26 S proteasome (17). The p27 subunit is also a component of the red cell 26 S proteasome (25). Insects do not have red blood cells since oxygen is supplied by diffusion from the spiracles via the hemolymph, so contamination of ISM extract with a red cell-derived modulator is not possible. The 220-kDa complex from intersegmental muscles is therefore the first 26 S proteasome activator described other than that in red blood cells.

The bovine 300-kDa “modulator” has recently been shown to promote the assembly of 26 S proteasomes from 20 S particles and the PA700 (19 S) regulator: the modulator increases the number of singly and doubly capped complexes (22), although activation by the modulator requires addition of only a single PA700 cap (23). Significantly, the increase in the concentration of the 220-kDa complex during development parallels the increase in the 26 S proteasome content of muscles (Figs. 2 and 3): the change in the 220-kDa complex in *Manduca* intersegmental muscles may facilitate the assembly of 26 S proteasomes in preparation for muscle cell death. However, ~3–5% of the S10b ATPase present in the 26 S proteasome (data not

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**TABLE II**

Properties of the human placental activator

| Glycerol gradient fractions | Volume | Activation |
|-----------------------------|--------|-----------|
|                             | %      | 3        |
|                             |        | 4        |
|                             |        | 5        |
| Proteasomes                 |        |          |
| Exp. A                      |        |          |
| Placental 26 S              | 70     | <0       |
| *Manduca* 26 S              | 70     | 122.6    |
| Exp. B                      |        |          |
| Placental 26 S              | 30     | 26.9     |
| Placental 26 S              | 70     | 106.9    |
| *Manduca* 26 S              | 70     | 350.2    |
| Human 20 S                  | 70     | 174.5    |
| Exp. C                      |        |          |
| Placental 26 S              | 70     | 25.0     |
| Human 20 S                  | 70     | <0       |

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**FIG. 7.** Identification of the p27 protein subunit in glycerol gradient fractions of Fraction II from human red cells. Human red blood cell lysate Fraction II was fractionated by glycerol gradient centrifugation. ○, chymotrypsin activity (arbitrary units); □, protein concentration (mg/ml). Pairs of fractions were pooled and subjected to Western analysis with different antibodies as indicated (see legend to Fig. 6 for details). *FII* (last lanes) shows a sample of total Fraction II protein, and *S10b* and *p27* (first lanes) show samples of His-tagged recombinant protein run as standards.
shown) is found in the 220-kDa complex, and ~90–95% of proteasomes in stage 7 ISM extracts are in 26 S complexes with correspondingly few 20 S particles (8). The data indicate that a complex containing a small fraction of total cellular S10b and S6\(^9\) ATPases can substantially activate (2–10-fold) preparations of Manduca 26 S proteasomes (Figs. 4 and 5 and Table I). Furthermore, the activator does not stimulate smaller (20 S particle-enriched) complexes within a proteasome preparation to a larger extent than larger (doubly capped 26 S particle-enriched) complexes (Table I, Experiment B). These observations are inconsistent with an activator mechanism involving reconstitution of depleted 26 S particles (5% of proteasomal particles) with their “missing” complement of S10b and S6\(^9\) ATPases. An alternative interpretation is that the activator is a normal physiological entity and that coordinated synthesis of the activator and 26 S proteasomes is required to permit full proteolytic activity of 26 S proteasomes during ISM cell death. The insect muscle 220-kDa activator is distinct from the 11 S (PA28) complex, which activates 20 S proteasomes in an ATP-independent manner (Table I).

The bead-immobilized Manduca activator complex retains the ability to activate the 26 S proteasome (Fig. 5). The gradient fractions in such a manner that their productive association is catalyzed. The extent of activation observed renders this mechanism unlikely. An alternative scenario could be that the immobilized activator is able to bind to either (a) a complete 26 S particle or (b) a 19 S particle that subsequently binds to a 20 S particle. It is possible that an S10b-S6\(^9\)-depleted 19 S particle is involved in these interactions, but the concentration of such particles in stage 7 ISM extracts is very low (8). In either case, the interaction with the 220-kDa complex increases proteasomal activity. This mechanism of activation by the immobilized activator is consistent with the fact that the full action of the modulator requires only a singly capped proteasome (23). Clearly, the 220-kDa complex and the modulator are related supramacromolecular structures.

A similar 220-kDa complex, containing the S10b and S6\(^9\) ATPases, but not the S6 or S7 ATPase, has been demonstrated in both insect and human tissues and cells (Figs. 6 and 9 and Table II), and the properties of the complex suggest that it is not an assembly intermediate or catalyst for 26 S proteasome formation (see above). However, a critical question is whether the activator or the modulator results from 26 S proteasome dissociation during tissue disruption and biochemical analyses. Several experimental approaches (e.g. the use of chaotropic salts) were adopted in studies on the bovine modulator to discount this notion (17). Similarly, in this work, omission of ATP from homogenization buffers did not result in the generation of more 220-kDa complexes from Manduca muscle 26 S proteasomes. However, comparison of the bovine modulator
and larger complexes containing the S7 ATPase (Fig. 8). In fresh tissue, the S7-containing complexes are not observed, and the majority of the S10b and S6' ATPases remain in the 26 S proteasomes. The 220-kDa complexes isolated under these latter conditions seem unlikely to be dissociated from placental 26 S proteasomes since the 220-kDa complex is able to activate *Manduca* 26 S proteasomes much more effectively than 26 S placental proteasomes isolated from the same glycerol gradient as the 220-kDa complex itself (Table II). We have already shown that *Manduca* stage 7 proteasome preparations comprise (90–95%) 26 S particles (8). Whatever the origin of the 220-kDa complex, it is clear from its ability to cross-activate 26 S proteasomes of different species that this proteasomal activator has been conserved during evolution.

S10b and S6' can form heterodimers or larger complexes with each other in vitro, but show much less tendency to interact with other proteasomal ATPases (1). Currently, we do not know whether the 220-kDa complex identified here comprises (a) several molecules of both S10b (44 kDa) and S6' (45 kDa) ATPases (24) in the form of, for example, a tetramer or hexamer or (b) one molecule of both these ATPases together with other unidentified subunits.

Recently, significant amounts of S7 ATPase have been found dissociated from the 26 S proteasome in human bladder carcinoma cells (T24 cells) during mitosis, associated instead with a nuclear protein containing leucine-rich heptad repeats (HEC (highly expressed in cancer cells)) that inhibits the degradation of mitotic cyclin B in vitro (30). The HEC protein peaks in the M phase of the cell cycle and may be part of a complex containing the anaphase-promoting complex/cyclosome that targets mitotic cyclins for degradation (31). The exact role for the S7 ATPase in such a complex is not clear. The S8 ATPase also interacts with HEC in a yeast two-hybrid screen (30) and has been found in a heterogeneous range of molecular species in HeLa cell nuclear extracts (32). Numerous other reports have been made of interactions of individual proteasomal ATPases with other proteins or complexes, particularly those involving transcription factors (32–37). Most of these interactions have been detected by yeast two-hybrid screens, and therefore, it is not known whether the ATPase involved is a single subunit or part of a complex with other ATPases.

The proteasomal ATPases belong to the AAA superfamily of ATPases involved in functions as diverse as membrane docking and fusion, peroxisome biogenesis, mitochondrial biogenesis, and proteasome regulation (38). The common molecular mechanism(s) by which the ATPases carry out these diverse functions are unknown, although protein unfolding and translocation may be common elements in these activities. Recently, one of the AAA ATPases has been shown to have molecular chaperone activity in that the ATPase domain recognizes and binds unfolded polypeptide substrates prior to their translocation to a peptidase domain for degradation (39). Proteasomal ATPases may act in an analogous manner. The potential role of individual ATPases, or species such as the 220-kDa activator complex described here, in substrate recognition for proteasome degradation or other processes remains to be elucidated. However, it is at least conceivable that the activator functions as an independent chaperone that can deliver substrates to the proteasome in a “processed” form, independent of the 19 S ATPases, which thus accelerates proteolytic degradation.

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