Proteasome activities decrease during dexamethasone-induced apoptosis of thymocytes

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The induction of apoptosis in thymocytes by the glucocorticoid dexamethasone was used as a model system to investigate whether there are changes in 20 S and 26 S proteasome activities during apoptosis. We observed that thymocytes contain high concentrations of proteasomes and that following treatment with dexamethasone, cell extracts showed a decrease in proteasome chymotrypsin-like activity which correlated with the degree of apoptosis observed. The decrease in chymotrypsin-like activity of 20 S and 26 S proteasomes was still apparent after these complexes had been partially purified from apoptotic thymocyte extracts and was therefore not due to competition resulting from a general increase in protein turnover. The trypsin-like and peptidylglutamylpeptide hydrolase activities of proteasome complexes were also observed to decrease during apoptosis, but these decreases were reversed by the inhibition of apoptosis by the caspase inhibitor benzoxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone. However, the chymotrypsin-like activity of proteasomes decreased further in the presence of the apoptosis inhibitor. Val-Ala-Asp-fluoromethylketone was found to inhibit the chymotrypsin- and trypsin-like activity of 26 S proteasomes in vitro. The decrease in proteasome activities in apoptosis did not appear to be due to a decrease in the concentration of total cellular proteasomes. Thus, the early decreases in 20 S and 26 S proteasome activities during apoptosis appear to be due to a down-regulation of their proteolytic activities and not to a decrease in their protein concentration. These data suggest that proteasomes may be responsible, in thymocytes, for the turnover of a protein that functions as a positive regulator of apoptosis.

INTRODUCTION

Apoptosis, or programmed cell death, is a highly regulated, genetically determined process by which unwanted cells are removed from multicellular organisms. Apoptosis is important during embryonic development and for metamorphosis, homeostasis of tissue mass and prevention of disease [1,2]. Cell death resulting from apoptosis is distinct from necrosis and is accompanied by a characteristic set of changes to the cell. These include cell shrinkage, chromatin condensation and fragmentation, break-up of the nucleus, blebbing of the cell and cleavage of DNA into nucleosomal-length fragments of 180–200 bp [3]. The observation that granzyme B in conjunction with perforin (a pore-forming protein) alone was sufficient to cause apoptosis of 20 S and 26 S proteasomes in vitro. The decrease in proteasome activities in apoptosis did not appear to be due to a decrease in the concentration of total cellular proteasomes. Thus, the early decreases in 20 S and 26 S proteasome activities during apoptosis appear to be due to a down-regulation of their proteolytic activities and not to a decrease in their protein concentration. These data suggest that proteasomes may be responsible, in thymocytes, for the turnover of a protein that functions as a positive regulator of apoptosis.

Among other proteases there is, in eukaryotes, some evidence for the involvement of calpains in apoptosis (reviewed in [9]). The present studies were undertaken to investigate the role of proteasomes in apoptosis. The 20 S proteosome is a large (≈ 700 kDa), hollow, cylindrical complex composed of four stacked rings each containing seven subunits. The subunits of the inner rings are associated with the proteolytic activities of the complex. The 20 S proteosome forms the catalytic core of the larger (≈ 2 MDa) 26 S proteasome (reviewed in [10]). The 26 S proteasome is the major extra-lysosomal machinery for the degradation of cellular proteins. The 26 S proteasome is responsible for the ATP-dependent degradation of proteins tagged for destruction by ubiquitin as well as non-ubiquitinated substrates. It is present in both the cytoplasm and nucleus of cells [11] and is responsible for the degradation of many important regulatory proteins with short half-lives. Some of these proteins, such as the tumour suppressor p53 [12] and nuclear factor κ-B (NFκ-B) [13], have themselves roles to play in apoptosis [14,15].

During the course of these studies a number of reports have implicated proteasomes in apoptosis from the observed effects of proteasome inhibitors [16–21]. However, they have produced contradictory evidence as to whether proteasomes are required to activate or to activate others by cleavage at sites following aspartate residues [6,9]. A number of peptide inhibitors of the caspases have been developed and benzoxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK), a cell-permeable, irreversible inhibitor of caspases, is a good inhibitor of apoptosis in many systems using a variety of apoptotic stimuli.

These data together suggest a key role for the caspases in apoptosis.

Abbreviations used: LSTR-NH-Mec, t-butoxycarbonyl-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin; Z-LLE-Nap, benzyloxycarbonyl-Leu-Leu-Glu-tetrapeptide; E-64, benzyloxycarbonyl-Leu-Leu-Val-Asp(OMe)-fluoromethylketone; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; VAD-FMK, Val-Ala-Asp-fluoromethylketone.

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for, or are protective against, apoptosis. The results of the studies presented here show that there is a decrease in proteasome activity in apoptotic thymocytes which correlates with the degree of apoptosis and which appears to be a specific down-regulation of proteasome activity. The commonly used apoptosis inhibitor Val-Ala-Asp-fluoromethylketone (VAD-FMK) (a reportedly specific caspase inhibitor) was also found to inhibit 26 S proteasome activity.

MATERIALS AND METHODS

Materials

20 S and 26 S proteasomes were purified from rat liver as previously reported [22–24]. Monoclonal antibodies, immuno-specific for proteasome subunit C2 (MCP20), were obtained from Dr. K. B. Hendil (August Krogh Institute, Copenhagen, Denmark) [25]. Dexamethasone, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-NH-Mec), t-butoxycarbonyl-Leu-Thr-Arg-7-amido-4-methylcoumarin (LSTR-NH-Mec) and benzoyloxycarbonyl-Leu-Leu-Glu-naphthylamide (Z-LLENap) were from Sigma Chemical Co. Z-VAD-FMK was purchased from Enzyme Systems Products and VAD-FMK was from Clontech. All other reagents were of analytical grade or better.

Thymocyte isolation and lysis

Thymocytes were isolated from 6–7-week-old male Fischer 344 rats as previously described [26]. Thymocytes were incubated for up to 6 h at approx. 20 × 10⁶ cells/ml in RPMI-1640 medium containing 10% (v/v) fetal calf serum in the presence or absence of 0.1 µM dexamethasone. Normal and apoptotic cells were quantified by flow cytometry as described [27]. Thymocytes were then sedimented at 1000 g for 5 min and as much as possible of the supernatant was removed. As rinsing the thymocytes was found to result in premature lysis and loss of cytoplasmic contents, and as the culture media did not contain any peptidase inhibitors, finding to result in premature lysis and loss of cytoplasmic contents, and as the culture media did not contain any peptidase inhibitors, the thymocytes were not washed further before lysis. Thymocytes (100 × 10⁶) were lysed in ice-cold homogenization buffer [20 mM Tris/HCl (pH 7.2)/0.1 mM EDTA/1 mM 2-mercaptoethanol/5 mM ATP/20% (v/v) glycerol, 0.04% (v/v) Nonidet P-40] by passage 25 times through a 21-gauge needle. The resulting lysates were placed in microfuge tubes and centrifuged at 13000 g at 4 °C for 15 min to remove insoluble material. The resulting high-speed supernatant fractions were placed on ice and assayed for protein concentration using protein assay reagent (Bio-Rad) and BSA as standard. Peptidase activities were measured in extracts or following gel-filtration chromatography.

Gel-filtration chromatography

Thymocyte extracts (equal amounts of protein loaded for each treatment, approx. 1.1 mg of protein per sample) were separated on a Superose 6 HR 10/30 gel-filtration column (Pharmacia) equilibrated in 20 mM Tris/HCl buffer, pH 7.2, containing 100 mM NaCl, 10% (v/v) glycerol and 5 mM ATP at a flow rate of 1 ml/min. Fractions (1 ml) were collected and immediately put on ice. Each fraction was assayed for protein concentration and for chymotrypsin-like peptidase activity as described below.

Assay of 20 S and 26 S proteasome activities

Assays were carried out in 50 mM Hepes, pH 8.0, containing 5 mM EGTA. For the determination of 20 S proteasome activities, SDS was added to a final concentration of 0.02%. At the protein concentrations used this amount of SDS was determined to activate the 20 S proteasome to its maximum activity while inhibiting 26 S proteasome activity. Thymocyte high-speed supernatant fractions (5 µl) or gel-filtration fractions (50 µl) were assayed using the substrates LLVY-NH-Mec (40 µM), LSTR-NH-Mec (40 µM) or Z-LLENap (400 µM) and assayed as described previously [23]. The activity against these substrates will be referred to as LLVY-NH-Mec, LSTR-NH-Mec and Z-LLENap activity below. Assay mixtures were incubated at 37 °C for 10, 25 or 60 min respectively.

Immunoblotting

Thymocyte high-speed supernatant fractions (40 µg) were separated on SDS/10% polyacrylamide gels according to the method of Laemmli [28], and electrophoretically transferred onto nitrocellulose. Immunoblotting was carried out as previously described [29] using MCP20, a monoclonal antibody specific for the C2 subunit of the 20 S proteasome, or polyclonal antibodies against 26 S proteasome subunit S12 (G. F. Mason, R. Z. Murray, D. Pappin and A. J. Rivett, unpublished work) using chemiluminescent detection (Renaisance, NEN) on Hyperfilm MP film (Amersham) according to the manufacturer’s instructions. Bands were quantified by scanning densitometry (GS-690 Imaging Densitometer, Bio-Rad). Proteasome subunits were quantified using known amounts of purified 20 S proteasome included in the gel to produce a standard curve.

Statistical analysis

Statistical analysis was carried out using Student’s t-test, P < 0.05 being taken as significant. Owing to unexplained variability of the specific peptidase activities in different batches of thymocytes, the results in some experiments are given as relative activity, i.e. as a percentage of the control activity. The latter values showed a high degree of consistency in different experiments.

RESULTS

Thymocytes contain high concentrations of 26 S proteasomes compared with other tissues

The relative protein levels of 26 S proteasomes were determined in a number of tissue types in order to find out whether thymocytes represented a good cell type in which to study the activities of the 26 S proteasome during apoptosis. Thymocyte high-speed supernatant fractions were electrophoresed on SDS/polyacrylamide gels, transferred to nitrocellulose membrane and subjected to immunoblotting with polyclonal antibodies raised against the S12 subunit of the regulatory complex. The S12 subunit is only found associated with 26 S proteasomes and no other complexes, and is not present as the free subunit (A. Z. Murray and A. J. Rivett, unpublished work). 26 S proteasomes were detected in every tissue tested and the highest concentration was present in thymus (Figure 1). High protein concentrations of the 26 S proteasome were also seen in spleen and brain, whereas muscle contained very little. The rank order of tissues for proteasome content in this study, while not dissimilar, is not identical with that previously reported by our laboratory [22] and others [30] using antibodies against core 20 S proteasome subunits.
Proteasome activity decreases in supernatant fractions of cells induced to apoptosis by exposure to dexamethasone

To determine whether proteasome activities altered during apoptosis, both 20 S and 26 S proteasome activities were measured in high-speed supernatant fractions from primary rat thymocytes induced to undergo apoptosis by the glucocorticoid agonist dexamethasone. Thymocytes which had been incubated for different times in either the presence or absence of dexamethasone (0.1 \( \mu \)M) and then lysed. Supernatants (approx. 10 \( \mu \)g) were incubated with substrate for 0.5 h and assayed in triplicate. Relative specific activity was calculated as nmol of LLVY-NH-Mec hydrolysed/h per \( \mu \)g of protein, expressed as a percentage of the 0 h control. The control activities were 0.442 ± 0.025 and 0.175 ± 0.008 nmol of LLVY-NH-Mec hydrolysed/h per \( \mu \)g of protein for 20 S and 26 S proteasomes respectively. Results are the means ± S.D. of experiments from three separate thymocyte preparations. *Statistically different from 0 h control. †Statistically different from the respective time control. ‡Statistically different from the 3 h treatment group.

### Table 1 Proteasome activities in crude extracts of dexamethasone-treated apoptotic thymocytes

<table>
<thead>
<tr>
<th>Time (h)/treatment</th>
<th>Apoptotic cells (% of total)</th>
<th>26 S Proteasome</th>
<th>20 S Proteasome</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>2.9 ± 1.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3 h control</td>
<td>7.6 ± 0.9</td>
<td>105 ± 4</td>
<td>64 ± 9.5*</td>
</tr>
<tr>
<td>3 h + dexamethasone</td>
<td>13.3 ± 1.2</td>
<td>89 ± 2.9†</td>
<td>45 ± 3.9†</td>
</tr>
<tr>
<td>6 h control</td>
<td>16.1 ± 0.8</td>
<td>104 ± 10</td>
<td>67 ± 4.3*</td>
</tr>
<tr>
<td>6 h + dexamethasone</td>
<td>38.5 ± 3.9</td>
<td>66 ± 10†‡</td>
<td>22 ± 3.2†‡</td>
</tr>
</tbody>
</table>

The activity of partially purified proteasomes decreases in cells induced to undergo apoptosis by exposure to dexamethasone

Following partial purification of 20 S and 26 S proteasomes from thymocyte high-speed supernatant fractions by Superose 6 gel filtration, there was a decrease in peptide hydrolysing activity in apoptotic samples compared with cells incubated without dexamethasone for a similar period of time (Table 2). However, the decrease in chymotrypsin-like activity with increasing apoptosis was not as marked with the 20 S proteasome as it was in high-speed supernatant fractions, unlike that observed for the 26 S proteasome, which was greater in the partially purified samples (Tables 1 and 2).
The decrease in proteasome activity is not due to increased protein degradation in apoptotic cells

It was possible that the observed decrease in LLVY-NH-Mec activity in high-speed supernatant fractions was due to the presence of higher amounts of proteins tagged for degradation through the ubiquitin–proteasome pathway that could act as competitive inhibitors. However, our results suggest otherwise, since the decrease in 26 S proteasome chymotrypsin-like specific peptidase activities remains after gel-filtration chromatography (Table 2) which separated the majority of cellular proteins from the 26 S and 20 S proteasomes, the bulk of protein eluting in two peaks (fractions 9–10 and 17–19) before and after the proteasome peaks (see Figure 2). The decrease was greater after a 6 h incubation compared with a 3 h incubation, reflecting a higher proportion of apoptotic cells.

Inhibition of apoptosis prevents the decrease in proteasome activities

The 20 S proteasome displays a number of distinct catalytic activities in addition to the chymotrypsin-like activity measured using LLVY-NH-Mec. In order to determine whether other activities of the proteasome are altered in apoptosis, the substrates LSTR-NH-Mec and Z-LLE-Nap were used to measure the trypsin-like and peptidylglutamylpeptide hydrolase activities respectively in the following experiments. If the decrease in the chymotrypsin-like activity of the proteasome is a regulated event occurring downstream of the onset of apoptosis it might be expected that the inhibition of apoptosis would prevent the decrease in proteasome activity following treatment of thymocytes with dexamethasone. Z-VAD-FMK, a specific inhibitor of the caspase family of enzymes, is known to inhibit apoptosis caused by a variety of initiating agents [6]. We therefore determined proteasome activities in extracts from thymocytes treated for 6 h with either dexamethasone alone or in combination with Z-VAD-FMK. The hydrolysis of both LLVY-NH-Mec and Z-LLE-Nap by thymocyte extracts was decreased following 6 h treatment of thymocytes with dexamethasone as compared with controls incubated for the same period of time for both the 20 S and 26 S proteasomes (Table 3). There was also a less pronounced decrease in activity against the substrate LSTR-NH-Mec. When thymocytes were treated with both dexamethasone and Z-VAD-FMK, however, the activities of the 20 S and 26 S proteasomes returned to control values for the trypsin-like and peptidylglutamylpeptide hydrolase activities measured by LSTR-NH-Mec and Z-LLE-Nap respectively. Indeed, some stimulation of activity was seen with Z-LLE-Nap. However, a different result was observed in the case of LLVY-NH-Mec, where the combined treatment with dexamethasone and Z-VAD-FMK caused an even greater decrease in activity than dexamethasone alone.

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Table 2 Activity of partially purified 26 S and 20 S proteasomes in apoptotic thymocytes

Extracts of thymocytes which had been incubated for 0, 3 or 6 h in the presence or absence of dexamethasone were fractionated by Superose 6 gel-filtration FPLC. Active fractions (1 ml) were pooled and assayed (50 µl) as described in the Materials and methods section. Relative specific activity was calculated as nmol of LLVY-NH-MEC hydrolysed/h per µg of protein, expressed as a percentage of the 0 h control. The control activities were 10.8 and 15.2 nmol of LLVY/h per µg of protein for 26 S and 20 S proteasomes respectively. Values are taken from a representative experiment where activities were measured in duplicate.

Table 3 Effect of a caspase inhibitor on dexamethasone-induced changes in proteasome activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (%)</th>
<th>LLVY-NH-Mec</th>
<th>LSTR-NH-Mec</th>
<th>Z-LLE-Nap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>26 S</td>
<td>20 S</td>
<td>26 S</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>77.9 ± 16.0*</td>
<td>86.4 ± 10.1</td>
<td>96.5 ± 14.7</td>
</tr>
<tr>
<td>Dexamethasone + Z-VAD-FMK</td>
<td></td>
<td>68.8 ± 11.6*</td>
<td>72.7 ± 13.8*</td>
<td>112 ± 21.7</td>
</tr>
</tbody>
</table>

*Statistically different from the control. †Statistically different from the dexamethasone alone group.

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Table 4 Effect of a caspase inhibitor on 20 S and 26 S proteasome activities

26 S and 20 S proteasomes were preincubated with VAD-FMK for 45 min at 25 °C and then assayed for activity using the indicated substrates as given in the Materials and methods section. Specific activity is in pmol of substrate hydrolysed/min per µg of protein. Values given are means ± S.D. (n = 3). *Statistically different from the control value.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LLVY-NH-Mec</th>
<th>LSTR-NH-Mec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.42 ± 0.52</td>
<td>6.01 ± 0.07</td>
</tr>
<tr>
<td>VAD-FMK</td>
<td>4.05 ± 0.07*</td>
<td>6.86 ± 0.36*</td>
</tr>
</tbody>
</table>

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The proportion of apoptotic cells after treatment with dexamethasone and Z-VAD-FMK caused an even greater decrease in activity than dexamethasone alone.
Caspase inhibitor VAD-FMK inhibits proteasome activity

It is possible that the large decrease in LLVY-NH-Mec activity seen on treating thymocytes with both dexamethasone and VAD-FMK is due to the peptide inhibitor inhibiting the chymotrypsin-like activity of the proteasome. We therefore tested whether VAD-FMK would inhibit the 20 S or 26 S proteasome in vitro. The activities of purified 26 S proteasomes were inhibited by approx. 25% by 100 μM VAD-FMK (Table 4). However, VAD-FMK was observed to stimulate the activity of the 20 S proteasome: LSTR-NH-Mec hydrolysis by 114 %, and LLVY-NH-Mec hydrolysis by 260 %.

The decrease in proteasome activities is not due to a decrease in proteasome concentration

The observed decrease in proteasome activities in apoptotic cells might simply reflect a decrease in the concentration of proteasomes. In order to test this we ran equal amounts of high-speed supernatant fractions from control and 6 h apoptotic thymocytes by SDS/PAGE and blotted the gels on to nitrocellulose membrane. The membranes were then probed with a monoclonal antibody raised against the 20 S proteasome subunit C2, MCP20. The C2 subunit is an α-type subunit present in all 20 S and 26 S proteasomes and is therefore a good determinant of total proteasome protein concentration. There was no significant change in the amount of proteasomes in dexamethasone- or dexamethasone/Z-VAD-FMK-treated cells compared with controls immunoblotted with MCP20 (results not shown).

DISCUSSION

Our present study shows that after 3 h treatment with dexamethasone there is a significant decrease in both 20 S and 26 S proteasome activities, which decrease even further by 6 h (Table 1). That these decreases in activity are observed in partially purified preparations also (Table 2), especially in the 26 S proteasomes, and reflect the degree of apoptosis, suggest that the observed changes in activity are due to specific regulation of proteasome activity. This is further suggested by our observations that the concentration of proteasome subunits does not change over the time period examined. In the cell the activity of proteasomes may be decreased even further by the movement of the protease to the periphery of the cell where it is associated with apoptotic blebs [31].

These data are in accord with a growing number of studies that have shown that treatment of various cell types with proteasome inhibitors causes them to undergo apoptosis [19–21,32–34]. However, this cellular response to proteasome inhibition is not unexpected, given the rapidly accumulating data supporting the role of the proteasome in the turnover of numerous short-lived regulatory proteins. Thus, indiscriminate inhibition of proteasomes is likely to lead to increased concentrations of key regulatory proteins resulting in apoptosis. Indeed, some proteins which are normally degraded by proteasomes, such as p53 and NFκ-B, have roles in apoptosis. The treatment of a number of cell types with proteasome inhibitors has been shown to result in an accumulation of p53 and apoptosis [19,21]. Furthermore, overexpression of a dominant-negative p53 in such cells prevented proteasome inhibitor-induced apoptosis, suggesting that the apoptosis produced was p53-dependent. However, glucocorticoid-induced apoptosis in thymocytes is also known to occur in a p53-independent manner [35].

In contrast with the above results, at least three studies have provided evidence that proteasome activity is required for apoptosis to occur. Proteasome inhibitors, including lactacystin, have been shown to inhibit apoptosis induced by a variety of agents in thymocytes and by growth-factor withdrawal in sympathetic neurons [16,18]. It might be argued that during apoptosis there could be an increase in the overall rate of protein turnover, which proteasome inhibitors prevent. However, no such increase was observed during thymocyte apoptosis [16]. Inhibition of specific proteasome substrates, however, may play a role. Proteasome inactivation by lactacystin in the T-cell hybridoma D0.11.10 leads to a decrease in the degradation of Iκ-B (inhibitor of NFκ-B) and a corresponding decrease in the translocation of transcriptionally active NFκ-B to the nucleus. This in turn is believed to result in a decrease of the expression of the cell death genes FasL and Fas, inhibiting anti-CD3-activation-induced apoptosis [36].

We treated thymocytes with Z-VAD-FMK to inhibit apoptosis and to determine whether changes in proteasome activities were downstream of caspase activation. However, the results were complicated by the fact that Z-VAD-FMK inhibited the chymotrypsin-like activity of the proteasome. During apoptosis in human monoblast U937 cells induced by tumour necrosis factor, lactacystin was observed to cause an increase in caspase-3 activity [37], and Bcl-2, an inhibitor of caspase-mediated apoptosis, was shown to inhibit apoptosis initiated by lactacystin in Ewing’s sarcoma cells, as measured by poly(ADP-ribose) polymerase cleavage [34]. Inhibition of proteasome activity has also been observed to prevent caspase-mediated poly(ADP-ribose) polymerase cleavage [16,18]. These data suggest that the proteasome elicits its effects upstream of the caspases in the apoptotic pathway. We observed significant changes in proteasome activity within 3 h of treatment with dexamethasone, but whether these changes precede any caspase activation is not known. It is possible that proteasomes might activate a caspase(s) at the top of the caspase cascade by limited proteolysis of its proregion such as is seen with Iκ-B [38]. Proteasomes can cleave after Asp in synthetic peptide substrates [23]. Furthermore, two-hybrid screening has shown that a protein with significant similarity to SEN3, the yeast homologue of the human S1 subunit of the 26 S proteasome, binds specifically to the tumour necrosis factor receptor upstream of its death domain [39], implying that the proteasome may play a very early role in apoptosis induced by tumour necrosis factor and Fas receptors. Other studies have shown a ubiquitin-conjugating enzyme to be able also to bind to the Fas receptor close to its death domain [40].

The apparent opposite effects of proteasome inhibitors on apoptosis described above seem irreconcilable. However, it should be noted that all the cell types where proteasome inhibition caused apoptosis are rapidly proliferating cells, whereas the thymocytes and neurons, which were protected from apoptosis by proteasome inhibitors, are not. Proteasomes have an essential role in cell-cycle regulation and their inhibition may have different effects in rapidly cycling and non-cycling cells. Thus, the effect of proteasome inhibitors may well depend on the inducing agent, the cell type and the specific apoptotic pathway activated. Indeed, proteasome inhibitors have been observed not to protect thymocytes against apoptosis induced by phorbol ester in combination with a calcium ionophore [16]. Also, interpretation of some of the above results is made more difficult by the fact that some of the proteasome inhibitors used have inhibitory properties against other proteases. The peptide aldehyde inhibitors, in particular, are not very specific for the proteasome at the concentrations used and will certainly inhibit calpains, which themselves have been suggested to play a role in apoptosis. This lack of specificity of protease inhibitors, especially small peptide-based inhibitors, has been demonstrated in this study by the finding
that the caspase inhibitor VAD-FMK also inhibits at least two activities of the 26 S proteasome, while stimulating these activities in the 20 S proteasome. Indeed, one group has reported purifying the proteasome based on its cleavage of the peptide substrate acetyl-Tyr-Val-Ala-Asp-methylcoumarin amide [41].

In conclusion, we show for the first time that proteasome activities decrease in an apparently regulated manner during dexamethasone-induced thymocyte apoptosis. Our data and activities of the 26 S proteasome, while stimulating these activities required to determine the exact role of the proteasome in apoptosis, which may depend on the cell type and/or the inducing agent.

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