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# Proteasomes play an essential role in thymocyte apoptosis

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**Cell death in many different organisms requires the activation of proteolytic cascades involving cytosolic proteases. Here we describe a novel requirement in thymocyte cell death for the 20S proteasome, a highly conserved multicatalytic protease found in all eukaryotes. Specific inhibitors of proteasome function blocked cell death induced by ionizing radiation, glucocorticoids or phorbol ester. In addition to inhibiting apoptosis, these signals prevented the cleavage of poly(ADP-ribose) polymerase that accompanies many cell deaths. Since overall rates of protein degradation were not altered significantly during cell death in thymocytes, these results suggest that the proteasome may either degrade regulatory protein(s) that normally inhibit the apoptotic pathway or may proteolytically activate protein(s) that promote cell death.**

*Keywords*: apoptosis/proteasome/thymocytes

## Introduction

Many signals have been described that induce apoptosis in thymocytes (Osborne *et al.*, 1994). These include exposure of cells to ionizing radiation, treatment with glucocorticoids, such as dexamethasone, and T cell receptor engagement. The latter mimics signals encountered when thymocytes undergo negative selection, the process by which potentially autoreactive T cells are deleted before exit into the peripheral circulation. While many of the signals that induce apoptosis in thymocytes have been identified, the molecular mechanisms that mediate these signals are poorly understood. A number of studies have shown that the signal transduction pathways described above are mediated, at least in part, by unique sets of genes (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Liu *et al.*, 1994; Woronicz *et al.*, 1994). For example, cell death induced by steroids requires the glucocorticoid receptor (Dieken and Miesfeld, 1992) while death by ionizing radiation requires p53 (Clarke *et al.*, 1993; Lowe *et al.*, 1993). In addition, negative selection in the thymus requires *nur77*, a member of the nuclear hormone receptor superfamily (Liu *et al.*, 1994; Woronicz *et al.*, 1994; Calnan *et al.*, 1995). The requirement for each of these genes is unique to the given induction pathway. Although multiple induction pathways lead to apoptosis in thymocytes, apoptosis is characterized in all cells by similar morphological features, suggesting that the final events in the execution phase of cell death may be shared.

One event shared by many, if not all, cell death pathways is the activation of a proteolytic cascade, mediated in part by a family of proteases known as the ICE proteases (reviewed by Martin and Green, 1995). Interleukin-1 converting enzyme (ICE) was first described as an activity necessary to cleave pro-interleukin (IL)-1β into active IL-1 (Thornberry *et al.*, 1992). Subsequent data demonstrated that *ced-3*, a gene required for all developmentally programed cell death in *Caenorhabditis elegans*, is homologous to ICE and suggested a more global role for this enzyme (Yuan *et al.*, 1993). Ectopic expression of ICE in fibroblasts and neuronal cells results in rapid induction of cell death, and inhibition of ICE activity by CrmA, an inhibitor of ICE encoded by cowpox virus, blocks these various types of cell death (Miura *et al.*, 1993, Wang *et al.*, 1994). ICE is now recognized as a member of a rapidly growing family of proteases that includes ICH-1/NEDD-2 (Kumar *et al.*, 1994; Wang *et al.*, 1994), CPP32/YAMA (Fernandes-Alnemri *et al.*, 1994; Tewari *et al.*, 1995), TX/ICH-2 (Faucheu *et al.*, 1995) and MCH-2 (Fernandes-Alnemri *et al.*, 1995), suggesting that different family members may participate in cell death pathways in specific cell types. The ICE family of proteases are zymogens which require proteolytic cleavage to become activated (Thornberry *et al.*, 1992), but precisely how ICE family members are activated is an area of intense investigation.

While the activation of ICE family members during cell death is well documented, few direct targets of the ICE family have been described. Several lines of evidence suggest that one important target of CPP32 is poly(ADP-ribose) polymerase (PARP), a 116 kDa nuclear enzyme that is involved in DNA excision repair (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995). PARP cleavage has been observed following the induction of apoptosis in many cell types (Kaufmann *et al.*, 1993). PARP is cleaved following DEVD, residues which resemble the sequence where ICE cleaves pro-IL-1β; however, recent data have identified CPP32, a close relative of ICE, as the protease that cleaves PARP (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). The functional consequences of PARP cleavage are not fully understood but, since cleavage severs the N-terminal zinc finger DNA binding domain of the protein from the C-terminal catalytic domain, it has been suggested that inactivation of DNA repair activity may result in apoptosis. Other proteins are known to be degraded following the induction of apoptosis, including lamin B1 (Neamati *et al.*, 1995), the 70 kDa component of U1 small nuclear ribonucleoprotein (Casciola-Rosen *et al.*, 1994)

and fodrin (Martin *et al.*, 1995). Whether these proteins are substrates of ICE family proteases remains to be determined.

The 26S proteasome is a conserved multicatalytic proteolytic complex present in all eukaryotic cells (Goldberg *et al.*, 1995; Coux *et al.*, 1996) that is responsible for the degradation of most cellular proteins (Rock *et al.*, 1994). The 26S proteasome is composed of a 20S catalytic core and associated regulatory proteins. This complex plays a critical role in the ubiquitin–proteasome-dependent proteolytic pathway, where it catalyzes the rapid degradation of proteins covalently linked to chains of ubiquitin. This pathway is highly regulated and selective, and regulates many important cellular processes such as cell cycle progression (Glotzer *et al.*, 1991; Pagano *et al.*, 1995). In addition, physiological signals can trigger the selective ubiquitination and rapid destruction of critical cell proteins. For example, the rapid degradation of the inhibitory protein, IκB, is essential for the activation of the important transcription factor, NFκB (Palombella *et al.*, 1994; Chan *et al.*, 1996). A number of inflammatory mediators [e.g. tumor necrosis factor (TNF)] signal IκB ubiquitination and degradation which allow NFκB movement into the nucleus resulting in transcription of various proteins important in the inflammatory response. Finally, the overall rate of protein breakdown in cells by the ubiquitin–proteasome pathway is also regulated precisely. The atrophy of mammalian skeletal muscle that occurs upon denervation, fasting or cancer cachexia is due primarily to enhanced proteolysis by this pathway (Wing *et al.*, 1995), and the dramatic atrophy and death of muscles in *Manduca sexta* is associated with activation of this degradative system (Dawson *et al.*, 1995; Jones *et al.*, 1995). In the proteasome, proteins are generally hydrolyzed to small peptides, most of which are degraded further to amino acids by cellular exopeptidases, while some are utilized in antigen presentation. However, it has also been demonstrated recently that the 26S proteasome can also catalyze limited proteolytic processing of inactive precursors to active forms. For example, the generation of the active form of NFκB involves ubiquitination and the proteolytic processing of inactive 105 kDa precursors to the active 50 kDa form by the 26S proteasome (Palombella *et al.*, 1994). Thus this structure may also play a role in the signal-induced proteolytic cascades that mediate cell death. Because of the involvement of proteolytic events in cell death and the known function of the proteasome in proteolytic processes, we have examined whether the proteasome also plays a central role in cell death. The data presented here demonstrate a requirement for the proteasome in apoptosis initiated in primary thymocytes by diverse stimuli.

## Results

### *Inhibition of the proteasome blocks cell death in thymocytes*

Several selective inhibitors of proteasome function that are valuable new tools for testing if this particle is involved in a cellular response recently have been identified (Rock *et al.*, 1994). The peptide aldehydes, MG132 (Cbz-leucinyl-leucinyl-leucinal or LLL), LLnL (*N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal, calpain I inhibitor) and LLM (*N*-acetyl-L-leucinal-L-leucinal-methional, calpain II inhibitor), readily enter cells and are known to suppress 26S proteasome activity reversibly in cultured cells. To determine if the proteasome plays a role in apoptosis, these compounds were added to primary thymocytes prior to or simultaneously with the induction of cell death by either ionizing radiation, glucocorticoids or the phorbol ester, PMA, which mimics T cell receptor engagement in thymocytes. Negative selection, the process by which autoreactive T cells are deleted from the thymus, occurs through engagement of the T cell receptor.

Figure 1 provides evidence that LLnL and LLM significantly inhibit apoptosis in thymocytes. Because these peptides can inhibit calpain as well as the proteasome (Rock *et al.*, 1994), we included in these experiments E64 ester, a cell-permeable inhibitor of the cysteine proteases calpain I and II, and the lysosomal proteases, cathepsins B, H and L. None of the concentrations of E64 tested blocked cell death in any of the conditions tested, further confirming the specificity of the proteasome inhibitors (Figure 2D). Identical results were obtained with Cbz-L-L-Y-$CHN_2$, another calpain inhibitor (data not shown). The inability of E64 to inhibit apoptosis suggests that the effects of LLnL or LLM are not mediated via calpain inhibition. It is also noteworthy that Ac-YVAD-CHO, a well characterized inhibitor of ICE (Thornberry *et al.*, 1992), does not prevent the induction of apoptosis. Other inhibitors of cysteine proteases such as antipain or leupeptin (which also inhibits trypsin-like serine proteases) also were ineffective in blocking cell death. In these experiments, LLnL was a more potent inhibitor of cell death, producing >70% inhibition of death induced by all three stimuli at 50 μM as compared with LLM which inhibits cell death by 45–55% at the same concentration. Accordingly, LLnL is a more potent inhibitor of proteasome function than LLM.

Because MG132, LLM and LLnL differ in their ability to inhibit the proteasome, we compared concentrations required to inhibit apoptosis in thymocytes treated as described above (Figure 2). MG132, the peptide most effective at inhibiting proteasome function (Palombella *et al.*, 1994), was most active in blocking apoptosis. This agent was able to inhibit 50% of death at 1 μM while 5–15 μM of LLnL or >50 μM LLM were required for similar inhibition. The effective concentration for each of these compounds was similar for all forms of cell death examined. Additional evidence indicating that these peptide aldehydes effectively block proteasome function includes our observation that treatment with these compounds also blocked the degradation of p53 (data not shown), a well characterized substrate of the ubiquitin–proteasome pathway (Scheffner *et al.*, 1990).

Recently, Fenteany *et al.* (1995) have demonstrated that lactacystin, a metabolite of *Streptomyces* originally isolated as an inhibitor of differentiation of a neuroblastoma line and cell cycle progression in several cell lines, is a specific inhibitor of proteasome activity. Lactacystin was shown to bind irreversibly and covalently to the N-terminal threonine of one of the β subunits of the proteasome (Fenteany *et al.*, 1995). It is known through X-ray crystallographic and mutagenesis studies that this position contains the active site nucleophile (Goldberg *et al.*, 1995; Löwe *et al.*, 1995; Seemüller *et al.*, 1995).

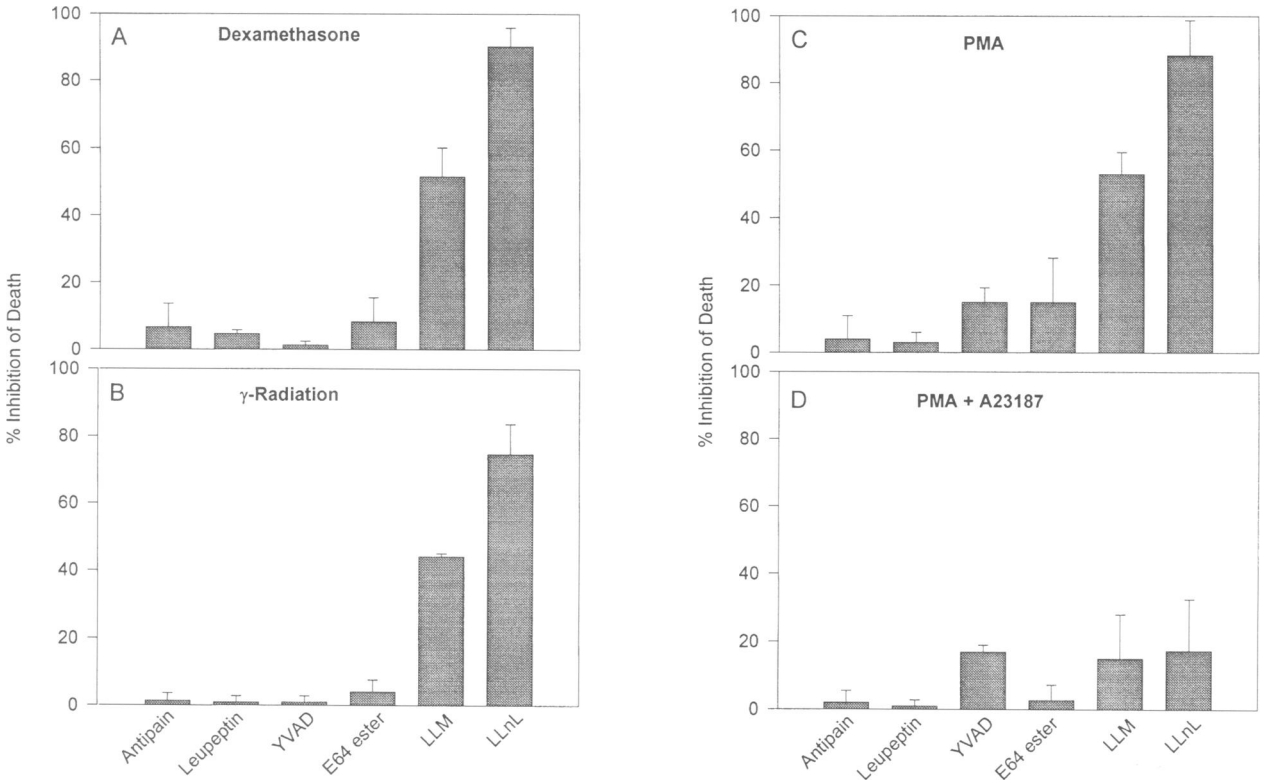


**Fig. 1.** Effect of a variety of protease inhibitors on thymocyte apoptosis. The protease inhibitors antipain (30 μM), leupeptin (30 μM), YVAD (30 μM), E64 ester (50 μg/ml), LLM (50 μM) and LLnL (50 μM) were added to thymocyte suspensions 1 h prior to treatment with (**A**) 5 μM dexamethasone, (**B**) 1000 Rads of γ-radiation, (**C**) 10 nM PMA or (**D**) 10 nM PMA + 500 nM A23187. At 8 h after induction of death, thymocytes were analyzed for FITC or YO-PRO-3 iodide staining on the FACScan. MTT assays were also used to measure viability. The percentage inhibition of death was calculated as follows: 100 – [% dead cells in inhibitor-treated population/% dead cells in control population×100].

Lactacystin has not been found to block the activity of other proteases including calpain, cathepsin B, chymotrypsin, trypsin and papain (Fenteany *et al.*, 1995). Thus lactacystin is a highly specific and irreversible inhibitor of the proteasome *in vitro* and in intact cells. In thymocytes, this compound was able to block apoptosis induced by radiation, dexamethasone or PMA (Figure 3). However, lactacystin did not block apoptosis induced by PMA + the calcium ionophore A23187 (see Discussion). The data obtained with lactacystin mimic those acquired using the peptide aldehyde inhibitors.

Peptide aldehydes are transition state analogs and thus are reversible inhibitors of proteases (Rock *et al.*, 1994; Löwe *et al.*, 1995). To test whether the inhibitory effects on apoptosis were reversible, thymocytes were treated with MG132 for 1 h, washed and exposed to dexamethasone for 8 h and assayed for the ability of these cells to undergo apoptosis. When compared with controls where MG132 was not removed, it is clear that the effects of MG132 are reversible (Figure 4). In similar experiments with lactacystin, which is an irreversible inhibitor of the proteasome, the inhibition of apoptosis was not reversed by removal of the drug (Figure 4). Taken together, these data provide further evidence that these agents are affecting apoptosis by specifically altering proteasome function.

In an attempt to define which step in the cell death pathway requires proteasome function, we asked when MG132 (1 μM) had to be present in order to inhibit apoptosis. Therefore, this agent was added to cells at various times following the induction of apoptosis by dexamethasone or ionizing radiation (Figure 5). These experiments demonstrate that cell death was inhibited when MG132 was added 1 h following induction of apoptosis. Partial inhibition was observed when MG132 was added at 3 h after induction. However, when MG132 was added 5 h after the induction of apoptosis, cell death was not blocked, indicating that the effects of the proteasome upon cell death must occur within the first 5 h following induction of death.

### *PARP cleavage is inhibited by LLM, LLnL and MG132*

One important enzyme that is inactivated proteolytically in apoptotic cells is the DNA repair enzyme PARP (Lazebnik *et al.*, 1994). To test whether inhibitors of proteasome activity also affect the apoptotic process before or after PARP cleavage, primary thymocytes were induced to die by either radiation or dexamethasone in the presence of LLM, LLnL or MG132 for 6 h. Whole cell extracts prepared from these cells were analyzed by SDS–PAGE, blotted onto nitrocellulose and probed with anti-PARP antibody, C-2-10. As shown in Figure 6, in unstimulated cells, intact 116 kDa PARP is detected but, after exposure to either radiation or dexamethasone, PARP was cleaved to an 85 kDa polypeptide. This cleavage was not inhibited detectably by 15 μM LLM, a concentration that is not effective at blocking cell death. However, 15 μM LLnL or 1 μM MG132 significantly blocked PARP cleavage. Lactacystin (10 μM) also blocked the cleavage of PARP (data not shown). E64 and dimethylsulfoxide (DMSO),

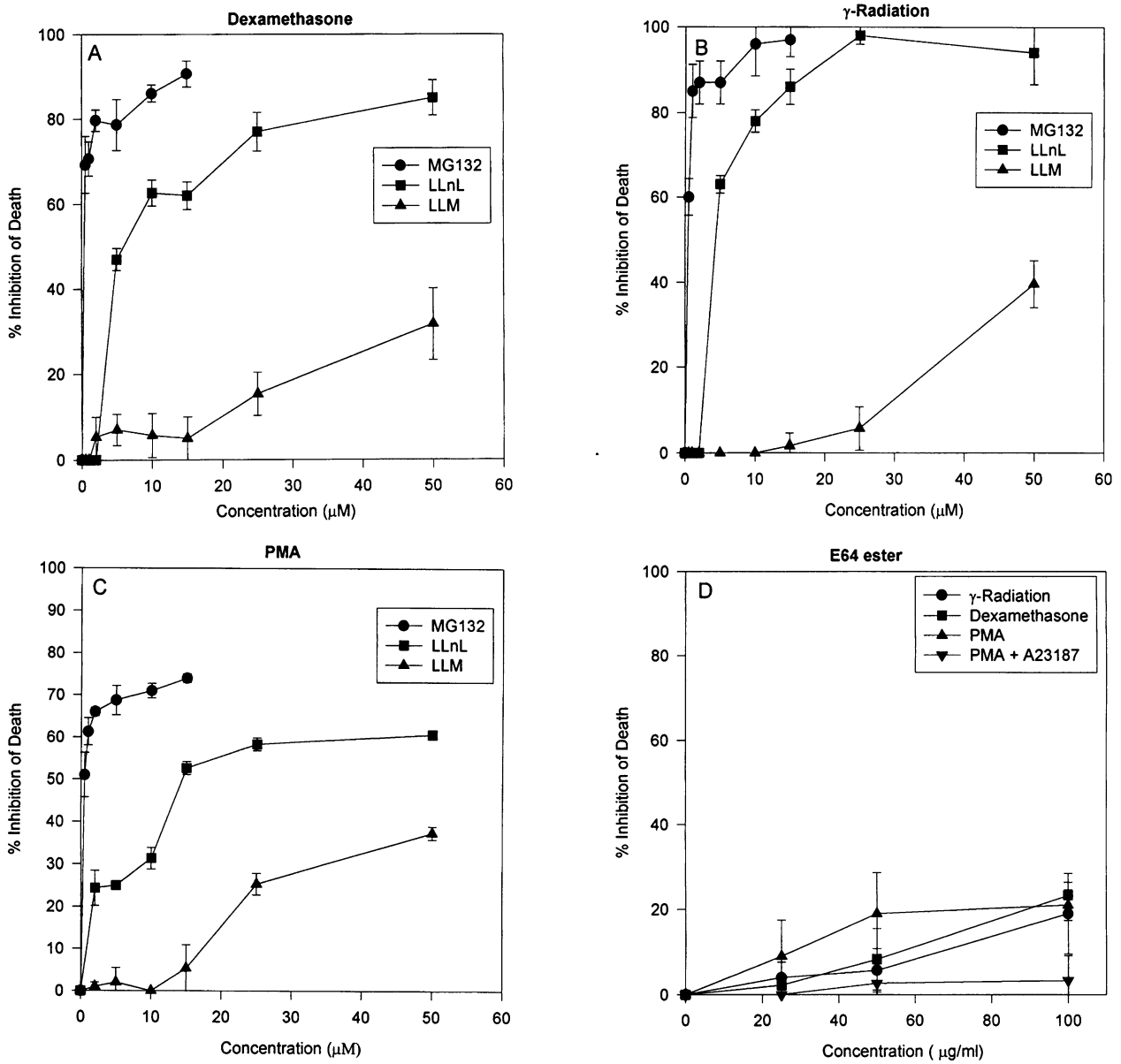


**Fig. 2.** Capacity of different concentrations of peptide aldehydes and E64 ester to inhibit thymocyte apoptosis. The protease inhibitors LLM, LLnL and MG132 were added to thymocyte suspensions at concentrations ranging from 500 nM to 50 μM at the same time that the cells were treated with (**A**) 5 μM dexamethasone, (**B**) 1000 Rads of γ-radiation or (**C**) 10 nM PMA. Cells were incubated with E64 ester at concentrations up to 100 μg/ml 1 h prior to treatment with (**D**) dexamethasone, γ-radiation, PMA or PMA + A23187. In all cases, thymocytes were analyzed for FITC or YO-PRO-3 iodide staining on the FACScan 8 h after induction to die. The percentage inhibition of death was calculated as indicated in Figure 1.

the diluent used to dissolve the peptide inhibitors, were used as controls, and neither blocked the cleavage of PARP.

### *Overall protein degradation rates do not increase with cell death*

The degradation of most intracellular proteins occurs through an extra-lysosomal pathway that involves proteasome activity, and the inhibition of the proteasome in lymphoblasts results in almost total cessation of intracellular protein degradation (Rock *et al.*, 1994). One possible explanation for the present findings is that cell death is accompanied by a generalized activation of the ubiquitin–proteasome pathway leading to an overall acceleration of protein breakdown that is inhibited by the proteasome inhibitors. To determine whether apoptosis in thymocytes is characterized by an increase in overall rates of protein degradation, cells were labeled with [$^3$H]tyrosine, either for 30 min to label short-lived proteins or for 6 h to label long-lived proteins, and resuspended in a large excess of non-radioactive tyrosine. Following labeling, cells were induced to die with either dexamethasone, radiation or PMA and incubated for either 1, 3 or 5 h in the presence of cold tyrosine. (Figure 7). In 1 h, ~15% of thymocyte proteins are degraded as determined by the amount of acid-precipitable radioactivity released. This rate is similar to that observed by Rock *et al.* (1994) who found ~20% of pulse-labeled proteins in B lymphoblastoid cell lines were degraded by 1 h. Thymocytes induced to die displayed a similar rate of protein degradation, indicating that induction of apoptosis is not accompanied by a general increased level of protein degradation. In similar studies, we determined that degradation of long-lived proteins

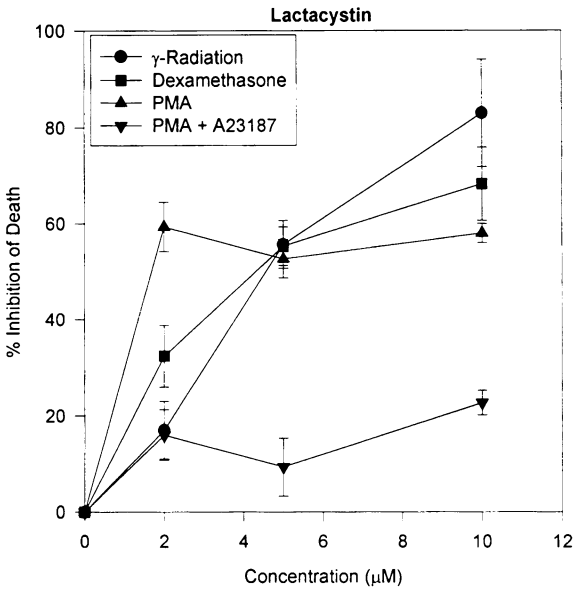


**Fig. 3.** Effect of lactacystin on thymocyte apoptosis induced with different stimuli. Thymocyte suspensions were treated with lactacystin at concentrations ranging from 2 to 10 μM simultaneously with treatment with 5 μM dexamethasone, 1000 Rads of γ-radiation, 10 nM PMA or 10 nM PMA + 500 nM A23187. Thymocytes were analyzed 8 h later for FITC or YO-PRO-3 iodide staining using the FACScan. The percentage inhibition of death was calculated as indicated in Figure 1.

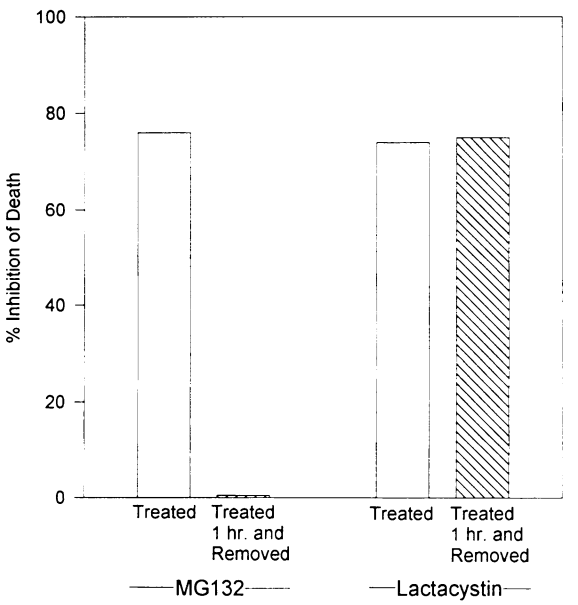


**Fig. 4.** Inhibitory effects of 1 h exposure and removal of MG132 and lactacystin on apoptosis in dexamethasone-treated thymocytes. Thymocyte suspensions were incubated for 1 h in the presence of 1 μM MG132, a reversible inhibitor of the proteasome, or 2 h in the presence of 10 μM lactacystin, an irreversible proteasome inhibitor. After incubation, one-half of the control cells and one-half of the inhibitor-treated cells were washed in PBS. The remaining cells from these two groups were unwashed. Washed and unwashed cells were treated with 5 μM dexamethasone and analyzed 8 h later for FITC or YO-PRO-3 iodide staining using the FACScan. The percentage inhibition of death was calculated as indicated in Figure 1.

followed the same kinetics in both normal thymocytes and those cells induced to undergo apoptosis (Figure 7B). Lastly, the addition of MG132 to cells during the determination of rates of proteolytic degradation significantly blocked the proteolysis of both short-lived and long-lived proteins, confirming that the degradation of proteins is due to the function of the proteasome (Table I). These results also confirm that the peptide inhibitors enter the cell and function as expected.

## Discussion

The intracellular events that mediate apoptosis are only partially characterized. In particular, it is still not clear if there are biochemical events common to all cell death pathways. Recently, it has become apparent that activation of an enzyme cascade is a common feature of many forms of cell death. Since the proteasome is the major site for degradation of nuclear and cytosolic proteins and has been shown to catalyze limited proteolytic processing of precursor proteins (Palombella *et al.*, 1994), it was reasonable to examine whether this proteolytic complex also plays an important role in apoptosis. The data presented here demonstrate that the proteasome is required for many forms of cell death in thymocytes. Our observations, in addition to data on cell death in neurons (Sadoul *et al.*, 1996), provide the first demonstration of a role for the proteasome in apoptosis. Additionally, because this requirement is observed in both thymocytes and neurons, the proteasome appears to play a role in many forms of programmed cell death.

Other investigators have suggested a role for proteases in T cell apoptosis. For example, Cohen and colleagues have suggested that activation of calpain is required in thymocyte apoptosis (Squire *et al.*, 1993). However the requirement for calpain originally was determined using LLnL, the peptide aldehyde subsequently shown by Rock *et al.* (1994) to block proteasome function in cell extracts and intact cells. Based on the present findings, it seems more likely that these authors were actually observing effects of inhibiting the proteasome in thymocyte apoptosis. Although the potencies of LLM and LLnL against calpains are very similar, their inhibitory effects on apoptosis were very different, and correlated with their relative potencies against purified 20S and 26S proteasomes and against proteolysis in intact cells (Rock *et al.*, 1994). Furthermore, in the present studies, several inhibitors of calpain I and II, including E64 ester, which is known to enter intact cells, leupeptin and antipain, had no inhibitory effect on apoptosis. Additionally, Sarin *et al.* (1995) also found recently that calpain inhibitors do not inhibit cell death in thymocytes, in agreement with our findings that calpain does not play a role in cell death in thymocytes. It is interesting that inactivation of these $Ca^{2+}$-activated proteases also did not block the cell death induced by $Ca^{2+}$ ionophore and PMA. Thus the rise in cytosolic $Ca^{2+}$ must trigger apoptosis by some other $Ca^{2+}$-dependent processes.

Our data indicate that calpain does not mediate apoptosis in thymocytes, but do the data support a role for the proteasome in apoptosis? In the experiments presented in Figure 1, we found that two protease inhibitors, LLM and LLnL, block thymocyte apoptosis, but these inhibitors are not specific inhibitors of the proteasome. To address the issue of specificity, we took advantage of the observation of Rock *et al.* (1994) that the $K_i$s of LLM, LLnL and

MG132 are almost identical against calpain, while the potency of these peptide aldehydes against the proteasome varies, with MG132 being the most potent, LLnL intermediate in potency and LLM the least potent. By measuring the dose–response of each of these inhibitors (Figure 2), we were able to demonstrate that their potency in inhibiting cell death mirrored that observed by Rock *et al.* (1994) against the proteasome. Although these data provided strong evidence for a role for the proteasome, the definitive experiment (Figure 3) demonstrated that lactacystin, a specific inhibitor of the proteasome, blocks thymocyte apoptosis. Therefore, these data provide convincing evidence that the proteasome plays an essential role in thymocyte apoptosis.

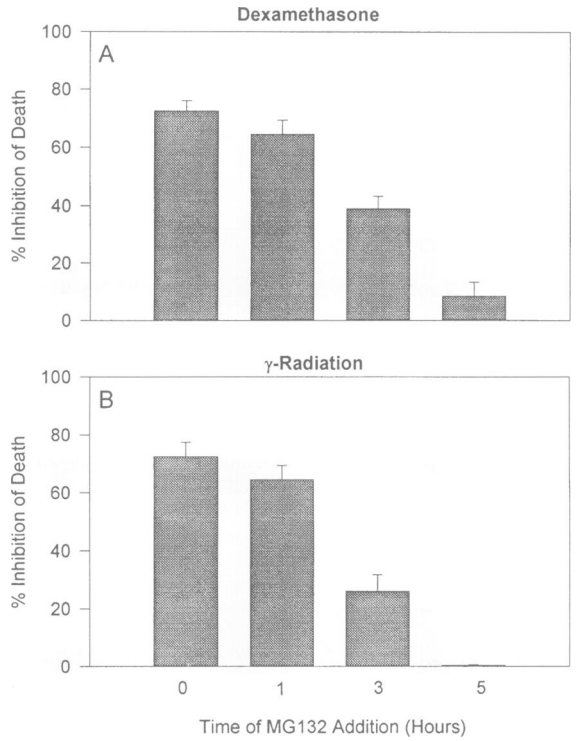


**Fig. 5.** Times when MG132 must be present to inhibit thymocyte apoptosis induced by dexamethasone or γ-radiation. Thymocyte suspensions were treated with either (**A**) 5 μM dexamethasone or (**B**) 1000 Rads of γ-radiation. Cells were exposed to 1 μM MG132 either simultaneously with treatment with dexamethasone or γ-radiation or 1, 3 or 5 h later. Cells were analyzed 8 h after induction of cell death for YO-PRO-3 iodide staining using the FACscan. The percentage inhibition of cell death was calculated as indicated in Figure 1.

In contrast to the cell death induced by X-rays, glucocorticoids or phorbol ester, thymocyte apoptosis induced by a combination of PMA and the calcium ionophore A23187 was not affected by lactacystin (Figure 3), LLM or LLnL (Figure 1). In related studies, we also have shown that staurosporine, a kinase inhibitor known to induce apoptosis in many different cell types (Raff *et al.*, 1993), can initiate apoptosis in the presence of the proteasome inhibitors (data not shown). Either PMA + A23187 and staurosporine induce cell death by a very different pathway or they activate the apoptotic pathway after the step requiring the proteasome. Thus, inhibition of the proteasome causes a specific inhibition of only certain activators of apoptosis in thymocytes. Furthermore, because the peptide inhibitors may be added as long as 3 h after the initiation of a death signal, the proteasome does not appear to play a role in the earliest events following the induction of apoptosis.

In some cell culture systems, proteasome inhibitors have been implicated in the induction of apoptosis (Imajohohmi *et al.*, 1995; A.Goldberg, unpublished observations). This is not surprising in light of the evidence for the requirement of the proteasome in cell cycle progression (Glotzer *et al.*, 1991; Pagano *et al.*, 1995). In an actively dividing cell population, inhibition of cell cycle progression might well be expected to be toxic in some instances. The data presented here demonstrate that, in the thymus, tissue that is not proliferating, short exposure to proteasome inhibitors is not toxic and, in fact, the proteasome is required for the induction of apoptosis. However, we have noted that prolonged exposure of thymocytes to these inhibitors over time increases the amount of background death, suggesting that prolonged inhibition of proteasome function might also be toxic to these cells (L.Grimm, unpublished observations).

The cleavage of PARP has been observed previously in

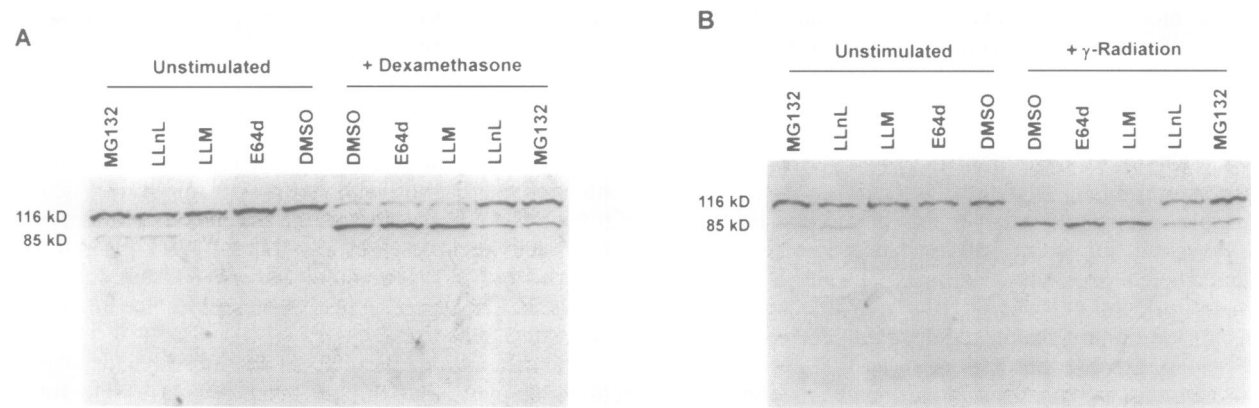


**Fig. 6.** Analysis of PARP cleavage in the presence of peptide aldehyde inhibitors and E64 ester. Thymocytes were pre-incubated for 1 h with DMSO, the solvent used for the inhibitors, E64 ester (50 μg/ml), LLM (15 μM), LLnL (15 μM) or MG132 (1 μM). Cells were then either unstimulated (lanes 1–5) or stimulated (lanes 6–10) with (**A**) 5 μM dexamethasone or (**B**) 1000 Rads of γ-radiation. At 6 h after the thymocytes were exposed to dexamethasone or γ-radiation, whole cell extracts were analyzed by Western blot for PARP cleavage. The mouse monoclonal antibody C-2-10 was used to detect PARP cleavage at a dilution of 1:10 000.

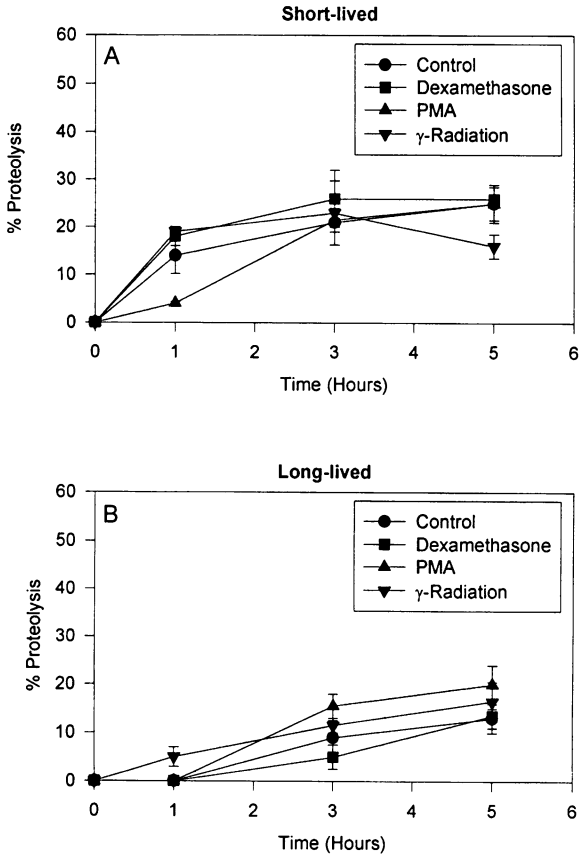


**Fig. 7.** Degradation of short-lived proteins in control versus dying thymocytes. Thymocytes were labeled with [$^3$H]tyrosine for (**A**) 30 min to label short-lived proteins or (**B**) 6 h to label long-lived proteins and subsequently resuspended in chase medium containing excess non-radioactive tyrosine. Immediately after suspension in the chase medium, cells were treated with 5 μM dexamethasone, 1000 Rads of γ-radiation or 10 nM PMA. TCA-precipitable radioactivity was measured at 0, 1, 3 or 5 h after induction of cell death. The percentage proteolysis was calculated as follows: 100 – [$^3$H-tyrosine c.p.m. from control and treated cell extracts at 1, 3 or 5 h/[$^3$H]tyrosine c.p.m. from control cell extracts at 0 h×100].

thymocytes induced to die with dexamethasone (Kaufmann *et al.*, 1993), but the data presented here represent the first report of a role for PARP cleavage in radiation-induced death in thymocytes and provide the first evidence that the proteasome is somehow required for PARP cleavage. It is well established that CPP32, an ICE family member, cleaves PARP (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995); therefore, the proteasome probably acts upstream in a pathway leading to CPP32 activation. Since ICE family members must themselves be cleaved to yield the active forms of the protease (Thornberry *et al.*, 1992), it is possible that the proteasome may act directly on proICE or proCPP32 or, alternatively, it may be involved indirectly in their activation (e.g. by degrading an inhibitor of their proteolytic processing). Our data suggest that ICE itself does not participate in thymocyte death, since the ICE inhibitor YVAD does not inhibit apoptosis in thymocytes (Figure 1). YVAD used at similar concentrations in motoneuron cell cultures inhibits cell death induced by growth factor withdrawal (Milligan *et al.*, 1995). Our data suggest that another ICE family member(s) participates in thymocyte apoptosis.

It is not yet clear what precise role the proteasome performs in cell death. It is possible that it cleaves a particular product that initiates a proteolytic cascade, for example the proteolytic processing of a proICE-like family member into an active protease. Proteasomal cleavage of an inactive precursor to yield a biologically active product has now been established for the proteolytic processing of an inactive p105 protein into the active p50 NFκB subunit. This event involves ubiquitination and limited proteolysis by the 26S proteasome and is sensitive to all of the inhibitors shown here to block apoptosis (Palombello *et al.*, 1994). In this process, the C-terminal sequence of the precursor is degraded, leaving the active N-terminal p50 fragment. Thus far, however, simple cleavage of a protein into two fragments by the proteasome has not been observed. It is even possible to speculate that NFκB, in addition to its role in triggering inflammation, may be one physiological target of the proteasome during apoptosis, since recent evidence has suggested a role for NFκB in cell death (Lin *et al.*, 1995). Another possible scenario is that the proteasome pathway degrades an inhibitor of apoptosis normally present to keep the cell death pathway in check (just as the onset of the inflammatory response is normally inhibited by IκB). The control and specificity of such a system could reside in the ability of the ubiquitin system to ubiquitinate a particular substrate, targeting it for destruction.

**Table I.** Degradation of short-lived and long-lived proteins 5 h after induction of cell death in the presence or absence of MG132

| Treatment | % Proteolysis of short-lived proteins | | % Proteolysis of long-lived proteins | |
|---|---|---|---|---|
| | –MG132 | +MG132 | –MG132 | +MG132 |
| Control | 25 (±3.5) | 4.0 (±2.0) | 13 (±2.0) | 0.0 (±0.0) |
| Dexamethasone | 26 (±1.0) | 1.0 (±1.0) | 14 (±3.5) | 4.0 (±1.0) |
| PMA | 25 (±4.0) | 1.3 (±1.3) | 20 (±4.0) | 4.3 (±1.3) |
| γ-radiation | 16 (±2.5) | 1.3 (±1.3) | 17 (±1.5) | 4.8 (±2.3) |

The observation that degradation of neither short-lived nor long-lived proteins is not increased during cell death directly suggests that the target of proteasomal degradation is a selected set of intracellular proteins. If there were a general increase in overall protein degradation during apoptosis (e.g. by an activation of the proteasome and the ubiquitin–proteasome pathway), then cell death might result from the destruction of various proteins necessary for cell viability. Such a generalized activation of this degradative pathway has been shown to be responsible for the muscle atrophy seen in denervation, fasting and other catabolic states (Wing and Goldberg, 1993; Medina *et al.*, 1995; Wing *et al.*, 1995) and also appears to accompany the massive atrophy and death of muscles of *M.sexta* (Dawson *et al.*, 1995; Jones *et al.*, 1995). However, we found that overall degradation rates do not increase during apoptosis. This supports a role for the proteasome during apoptosis in the degradation of selected regulatory proteins.

Lastly, it has been shown in both vertebrates and invertebrates that the functional capacity and subunit composition of the proteasome complex may vary under different physiological conditions. For example, when mammalian cells are treated with γ-interferon, which enhances antigen presentation, three new 20S subunits are

produced that specifically replace constitutively expressed subunits (Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993). Their incorporation results in the preferential production of the types of peptide that are transported selectively into the endoplasmic reticulum and strongly bind MHC class I molecules. Perhaps more germane to the findings presented here, two groups have reported the induction of new proteasomal subunits in *M.sexta* muscles following the induction of cell death (Dawson *et al.*, 1995; Jones *et al.*, 1995). It remains unclear how these major changes in proteasome composition influence proteasome function. They may be important in the dramatic loss of cell mass in these muscles or may be related to the proteolytic events leading to cell death. It will be interesting to determine if, during cell death in thymocytes, the structure of the proteasome changes or the control of substrate degradation is at the level of ubiquitination. It is known in several cell types that specific physiological conditions lead to the appearance of specialized enzymes for ubiquitination (e.g. in reticulocyte development) (Wefes *et al.*, 1995).

In conclusion, the present study, and that of Sadoul *et al.* (1996), provide compelling evidence for a role for the proteasome in apoptosis. Thus this critical pathway is found to regulate not only cell proliferation and homeostasis but also programmed cell death. The identification of the precise cellular targets of the proteasome during apoptosis will further enhance our understanding of proteolytic cascades and their contribution to cell death.

## Materials and methods

### Reagents

The protease inhibitors Ac-LLM-CHO, Ac-LLnL-CHO and E64 ester (E64d) were purchased from Sigma Chemical Co. (St Louis, MO). Dexamethasone, PMA, A23187, MTT and FITC were also purchased from Sigma. YO-PRO-3 iodide was purchased from Molecular Probes (Eugene, OR). The RPMI-1640-Select-Amine kit was purchased from GIBCO BRL (Gaithersburg, MD). [$^3$H]Tyrosine is a product of DuPont NEN (Boston, MA). Ac-YVAD-CHO, Ac-FRVR-CHO (antipain) and Ac-LLR-CHO (leupeptin) were a kind gift from Dr Kevin Tomaselli. MG132 (Cbz-LLL-CHO) and lactacystin were kindly provided by ProScript, Inc. (formally known as Myogenics).

### Treatment of thymocyte suspensions with protease inhibitors

Thymuses were isolated from 3–5-week-old BALB/c mice. Thymuses were placed into 1× phosphate-buffered saline (PBS) and gently ground between the frosted ends of wetted microscope slides to release the thymocytes from the thymic matrix. The thymocytes were pelleted by centrifugation at 200 *g* for 10 min. The pellets were resuspended in RDG + 10% horse serum at a final density equal to $5.0\times10^6$ cells/ml.

All protease inhibitors were suspended in DMSO and added to cells at a final concentration of <0.2%. Control cells received only DMSO. The inhibitor stocks were stored at the following concentrations: LLM, LLnL and MG132 at 50 mM; antipain, leupeptin, and YVAD at 20 mM; E64 ester at 25 mg/ml, and lactacystin at 10 mM. Protease inhibitors were added either 1 h prior to or simultaneously with the inducers of cell death.

### Cell death induction and analysis

Thymocytes were induced to die using four different treatments, including dexamethasone, γ-radiation, PMA or PMA + A23187. Cell suspensions were treated with 5 μM dexamethasone, 10 nM PMA alone or 10 nM PMA in the presence of 500 nM calcium ionophore (A23187). Thymocytes were irradiated with 1000 Rads in a Gammator-50 that releases 7.5 Rads/s.

Thymocyte suspensions were analyzed for viability using three different procedures. The MTT assay assesses the activity of mitochondrial dehydrogenases in living cells. A total of $1.7\times10^6$ cells (30 μl) were mixed thoroughly with 3.0 μl of MTT (5.0 mg/ml stock solution). Samples were incubated in 5.0% $CO_2$ at 37°C for 2 h. An equal volume of acidic isopropanol (0.04 M HCl in absolute isopropanol) was added to each sample and mixed thoroughly to dissolve any precipitate. Samples were spun at 7000 *g* for several seconds. Absorbances of the supernatants were measured at a wavelength of 590 nm on an Elisa reader (Cambridge Technologies).

FITC is a cytoplasmic stain that assesses membrane permeability. FITC was added to thymocyte suspensions at a final concentration equal to 25 μg/ml. Cells were incubated with FITC in a 5.0% $CO_2$ incubator at 37°C for 15 min. Cells were washed three times in 1× PBS prior to fixation in 1.0% formalin. Cells were analyzed for FITC staining using the FACScan.

YO-PRO-3 iodide is a DNA binding stain that assesses membrane permeability. Cells were resuspended in PBS + YO-PRO-3 iodide; 0.5 μl of YO-PRO-3 iodide were added to every 1.0 ml of PBS. Cells were analyzed immediately for staining using the FACScan.

### Analyzing inhibition of death after limited exposure to MG132 and lactacystin

One population of thymocytes was treated for 1 h with MG132, and a second population was treated for 2 h with lactacystin. Each population of cells was divided into two groups. One group remained exposed to the inhibitors, while the second group was washed three times in 1× PBS to remove the inhibitors. All groups of cells were treated with either dexamethasone or γ-radiation.

### Analyzing the potency of MG132 at various times after the induction of cell death

For analysis of the time when MG132 must be present to block cell death, cells were treated with MG132 at various times after the induction of cell death from exposure to either 5 μM dexamethasone or 1000 Rads of γ-radiation. MG132 was added either simultaneously with dexamethasone or γ-radiation treatment or 1, 3 or 5 h later.

### Assays of intracellular protein degradation

Three different media were required for analysis of protein degradation. All media were made from the RPMI-1640-Select-Amine kit. Each medium was composed of RPMI-1640 + 10% fetal calf serum and varying concentrations of non-radioactive tyrosine. Tyrosine was omitted from the medium used to label short-lived proteins (called short-term labeling medium). The medium used to label long-lived proteins (called long-term labeling medium) contained 10% of the normal amount of tyrosine (2.0 μg/ml). The chase medium, used to prevent reincorporation of [$^3$H]tyrosine released during proteolysis, contained 70 μg/ml tyrosine [i.e. the amount of tyrosine included in standard medium (20 μg/ml) plus an additional 50 μg/ml].

To label short-lived proteins, thymocytes were incubated for 30 min in short-term labeling medium containing 5.0 μCi/ml [$^3$H]tyrosine. Cells were washed and resuspended in chase medium (final density equal to $5.0\times10^6$ cells/ml). Cells (500 μl in triplicate) were stimulated to die by treatment with either dexamethasone, γ-radiation or PMA. At 0 or 1, 3 or 5 h after induction of cell death, cells were collected by centrifugation at 200 *g* for 10 min. One-half of the 5 h samples were treated with 1 μM MG132 simultaneously with exposure to the cell death inducers. Then 20% trichloroacetic acid (TCA) (500 μl) was added in the presence of 0.1 mg/ml bovine serum albumin (BSA) (500 μl). After incubation for 30 min on ice, samples were collected onto glass microfiber filters (Whatmann GF/C) using a vacuum manifold (Millipore). Samples were washed twice with 5.0 ml of 10% TCA and twice with 5.0 ml of 95% ethanol. The amount of TCA-precipitable radioactivity was measured in a liquid scintillation counter (Beckman).

The labeling of long-lived proteins was achieved by incubation of the thymocytes in long-term labeling media containing 5.0 μCi/ml [$^3$H]tyrosine for 6 h. Samples were processed exactly as described for the labeling of short-lived proteins.

### Western blots

Whole cell lysates were resolved on a 10% SDS gel, transferred to nitrocellulose and processed as previously described (Harlow and Lane, 1988). The anti-PARP mouse monoclonal antibody, C-2-10, was used at a dilution of 1:10 000. This antibody recognizes an epitope located between amino acids 216 and 375 in the N-terminal portion of the protein (Lamarre *et al.*, 1986). The secondary antibody was a sheep anti-mouse antibody conjugated to horseradish peroxidase used at a dilution of 1:10 000. Signals were visualized using ECL (Amersham).

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