p53-dependent Induction of Apoptosis by Proteasome Inhibitors

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Proteolysis by the ubiquitin/proteasome pathway controls the intracellular levels of a number of proteins that regulate cell proliferation and cell cycle progression. To determine whether this pathway of protein turnover was also linked to apoptosis, we treated Rat-1 and PC12 cells with specific proteasome inhibitors. The peptide aldehydes PSI and MG115, which specifically inhibit the chymotrypsin-like activity of the proteasome, induced apoptosis of both cell types. In contrast, apoptosis was not induced by inhibitors of lysosomal proteases or by an alcohol analog of PSI. The tumor suppressor p53 rapidly accumulated in cells treated with proteasome inhibitors, as did the p53-inducible gene products p21 and Mdm-2. In addition, apoptosis induced by proteasome inhibitors was inhibited by expression of dominant-negative p53, whereas overexpression of wild-type p53 was sufficient to induce apoptosis of Rat-1 cells in transient transfection assays. Although other molecules may also be involved, these results suggest that stabilization and accumulation of p53 plays a key role in apoptosis induced by proteasome inhibitors.

Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, growth factor deprivation, and abnormal expression of oncogenes or tumor suppressor genes (1–3). Apoptosis induced by these various agents appears to be mediated by a common set of downstream elements that act as regulators and effectors of apoptotic cell death. In many cases, apoptosis requires the p53 tumor suppressor protein (4). Overexpression of p53 is not only induced by several cell death stimuli but is itself sufficient to induce apoptosis in gene transfer assays (5). Apoptosis is also regulated by members of the Bcl-2 family (2, 6), which act upstream of a family of cysteine proteases known as the interleukin-1β converting enzyme (ICE)1 protease family (3, 7). Members of the ICE family, which are activated by proteolytic cleavage of proenzymes, appear to act as common executioners of apoptosis induced by a variety of cell death stimuli.

The ubiquitin/proteasome pathway is a major pathway of proteolysis in eukaryotic cells and may contribute to controlling the intracellular levels of a variety of short-lived proteins (8–10). The proteasome is a large multicatalytic complex that catalyzes the degradation of ubiquitinated cellular proteins. Substrates of the ubiquitin/proteasome pathway include a number of cell regulatory molecules, such as cyclins, the Myc oncogene protein, and p53, and the regulated degradation of these molecules has been linked to the control of cell proliferation and cell cycle progression (8–10).

By controlling the intracellular levels of such proteins, the activity of the ubiquitin/proteasome pathway might also be linked to apoptosis. In the present study, we have tested this possibility and demonstrate that inhibition of the chymotrypsin-related activity of the proteasome induces apoptosis of proliferating Rat-1 cells and of PC12 cells. This induction of apoptosis is associated with increased intracellular levels of p53 and is blocked by dominant negative p53 mutants, suggesting that it results from inhibition of p53 degradation.

Materials and Methods

Cell Cultures—Rat-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. PC12 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 5% horse serum. Cells overexpressing Bcl-2 and CrmA were kindly provided by J. Yuan (Massachusetts General Hospital). Cells overexpressing dominant-negative p53 were obtained by transfection with plasmids expressing the p53 mutant Val1243→Ala from a cytomegalovirus promoter (11).

Induction of Apoptosis by Proteasome Inhibitors—N-Benzoyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal (PSI) and carbobenzoxyl-leucinyl-leucinyl-norvalinal-II (MG115) were purchased from the Peptide Institute Inc. and diluted in Me2SO. The alcohol analog of PSI, N-benzoyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal, was kindly supplied by S. Wilk (Mount Sinai School of Medicine, New York, NY). Calpain inhibitor II was purchased from Boehringer Mannheim. The peptide aldehydes were added to cultures, whereas control plates were treated with the diluent. Treated cells were collected by centrifugation, and cytoplasmic DNA was isolated, electrophoresed in 1.8% agarose gels, and visualized by ethidium bromide staining (12). Cytological characterization of apoptotic cells was performed by staining with propidium iodide and TUNEL assay (Boehringer Mannheim).

Immunoblot Analysis—Cell lysates (20 μg) were electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary monoclonal antibodies against p53, p21, and Mdm2 were purchased from Santa Cruz Biotechnology Inc. and diluted in Me2SO. The alcohol analog of PSI, N-benzoyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal, was kindly supplied by S. Wilk (Mount Sinai School of Medicine, New York, NY). Calpain inhibitor II was purchased from Boehringer Mannheim.

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Induction of Apoptosis by Proteasome Inhibitors

Fig. 1. Induction of apoptosis by proteasome inhibitors. Cells were treated for 4 h either with the diluent Me2SO or with the indicated concentrations of inhibitors. Apoptosis was assayed by oligonucleosomal fragmentation of DNA. A, proliferating Rat-1 fibroblasts (R) or PC12 pheochromocytoma cells were treated with Me2SO or the proteasome inhibitor PSI. B, proliferating Rat-1 fibroblasts were treated with PSI (lanes 1 and 2), the alcohol analog of PSI (Pep. Mod., lane 3), the proteasome inhibitor MG115 (lane 4), the lysosomal inhibitor NH4Cl (lane 5), calpain II inhibitor (lane 6), or Me2SO (lane 7). C, Rat-1 cells were treated with PSI (lane 1), the alcohol analog of PSI (lane 2), or Me2SO (lane 7). Transfected Rat-1 cells expressing CrmA (lanes 3 and 4) or Bcl2 (lanes 5 and 6) were treated with PSI and MG115. D, Rat-1 cells were growth-arrested by incubation in medium containing 0.5% calf serum for 36 h and treated with Me2SO (lane 1) or MG115 (lane 2). Parallel cultures of proliferating Rat-1 cells were treated with MG115 (lane 3) or Me2SO (lane 4). Proliferating PC12 cells were treated with MG115 (lane 5). Nonproliferating differentiated PC12 cells were obtained by treatment with nerve growth factor (100 ng/ml) in medium containing 0.5% horse serum for 8 days and treated with MG115 (lane 6) or Me2SO (lane 7). DMSO, Me2SO.

Both Rat-1 and PC12 cells underwent apoptosis following treatment with PSI at concentrations of 15 or 25 μM (Fig. 1A). Apoptosis was similarly induced by treatment of Rat-1 cells with 30 μM MG115 (Fig. 1B, lane 4). These data were confirmed by the TUNEL assay, which provides an in situ assay for DNA fragmentation. In a typical experiment, 10–15% of cells were TUNEL positive following 5 h of treatment with PSI compared with 1–2% of untreated control cells (data not shown).

In contrast to the activity of PSI and MG115, apoptosis was not induced by treatment with NH4Cl, which nonspecifically inhibits lysosomal proteolysis, or with a specific inhibitor of calpain II (Fig. 1B, lanes 5 and 6). Thus, inhibition of lysosomal proteases did not lead to apoptosis. As a further specificity control, cells were treated with an alcohol analog of PSI, which only weakly inhibits proteasome function (14). The alcohol analog failed to induce apoptosis of Rat-1 cells (Fig. 1B, lane 3, and C, lane 2), indicating that apoptosis induced by PSI and MG115 resulted from specific inhibition of the proteasome.

Apoptosis induced by a variety of agents can be inhibited by overexpression of Bcl-2 or by inhibition of ICE family proteases. We therefore tested the effects of Bcl-2 and CrmA, a cowpox virus-encoded ICE inhibitor, on apoptosis induced by proteasome inhibitors. Transfected Rat-1 cells overexpressing either Bcl-2 or CrmA were resistant to treatment with PSI and MG115 (Fig. 1C), indicating that apoptosis induced by these proteasome inhibitors was regulated by Bcl-2 and mediated by ICE family proteases.

Induction of apoptosis by proteasome inhibitors contrasts with recent results, indicating that inhibition of the proteasome prevents apoptosis of thymocytes and sympathetic neurons (24, 25). It appeared possible that this difference was due to the fact that the cells used in the present study were actively proliferating, in contrast to terminally differentiated thymocytes and neurons. We therefore tested the effects of proteasome inhibitors on nonproliferating Rat-1 cells, which had been rendered quiescent by serum deprivation, and on PC12 cells, which had been induced to differentiate by treatment with nerve growth factor. Treatment with MG115 failed to induce apoptosis of quiescent Rat-1 cells but was still able to induce apoptosis of nonproliferating differentiated PC12 cells (Fig. 1D). Thus, cell proliferation was not the sole determinant of cellular sensitivity to proteasome inhibitors.

Accumulation of p53 in Cells Treated with PSI and MG115—

Fig. 2. Accumulation of p53 in cells treated with proteasome inhibitors. A, proliferating Rat-1 cells were treated with Me2SO, PSI, calpain II (CII) inhibitor, or MG115 for the indicated times. Cell lysates were immunoblotted with anti-p53 monoclonal antibody. B, proliferating Rat-1 and PC12 cells were treated with Me2SO (DMSO), PSI, MG115, or the alcohol analog of PSI (Pep. Mod.) for 4 h.

dylglutamyl-peptide hydrolizing activity (13). The chymotrypsin-related activity is specifically inhibited by the peptide aldehydes PSI and MG115 (14), so we initially tested the possibility that these inhibitors would induce apoptosis.
p53 is required for apoptosis in a number of models and stabilization of p53 leads cells to undergo apoptosis [4, 5]. Degradation of p53 occurs through the ubiquitin/proteasome pathway [19, 20]. If this involved the chymotrypsin-related function of the proteasome, PSI and MG115 might induce apoptosis by inhibiting p53 degradation.

We therefore tested the levels of p53 in Rat-1 and PC12 cells following treatment with proteasome inhibitors (Fig. 2). In both cell types, PSI and MG115 lead to the accumulation of p53, which could be detected as early as 2 h after initiation of treatment (Fig. 2A, lane 2). In contrast, treatment with the lysosomal protease inhibitor calpain II did not result in p53 accumulation (Fig. 2A, lanes 6 and 7), nor did treatment with the alcohol analog of PSI (Fig. 2B, lanes 2, 3, 7, and 8). Most of the p53 accumulated following treatment with the proteasome inhibitors migrated at its normal electrophoretic mobility, corresponding to 53 kDa, and therefore appeared to be nonubiquitinated. However, higher molecular mass bands that reacted with anti-p53 monoclonal antibody were also observed after treatment with proteasome inhibitors (for example, Fig. 2A, lanes 2–4, 8, and 9). These bands might correspond to accumulation of ubiquinated p53 complexes.

p53 is a transcriptional activator that stimulates expression of several genes, including the cyclin-dependent kinase inhibitor p21 and the p53 negative regulator Mdm2 [21]. To determine whether the p53 accumulated in response to treatment with proteasome inhibitors was functional, we assayed expression of these genes in cells treated with MG115. Treatment with MG115-induced expression of both p21 (Fig. 3A) and Mdm-2 (B). Positions of p21 and Mdm-2 are indicated by arrows. DMSO, Me2SO.

The results of the present study demonstrate that treatment of proliferating Rat-1 cells and of PC12 cells with inhibitors of the chymotrypsin-like function of the proteasome induces apoptosis. A number of previous investigations have demonstrated that the peptidyl aldehydes employed in this study are specific inhibitors of the proteasome chymotrypsin-related activity [14–18]. In contrast, neither inhibitors of lysosomal proteases nor a peptide alcohol analog that is ineffective as a proteasome inhibitor was able to induce apoptosis. It thus appears that inhibition of this function of the proteasome specifically induces apoptosis, linking the ubiquitin/proteasome pathway of protein degradation to regulation of apoptotic cell death.

**DISCUSSION**

The results of the present study demonstrate that treatment of proliferating Rat-1 cells and of PC12 cells with inhibitors of the chymotrypsin-like function of the proteasome induces apoptosis. A number of previous investigations have demonstrated that the peptidyl aldehydes employed in this study are specific inhibitors of the proteasome chymotrypsin-related activity [14–18]. In contrast, neither inhibitors of lysosomal proteases nor a peptide alcohol analog that is ineffective as a proteasome inhibitor was able to induce apoptosis. It thus appears that inhibition of this function of the proteasome specifically induces apoptosis, linking the ubiquitin/proteasome pathway of protein degradation to regulation of apoptotic cell death.
Induction of apoptosis by proteasome inhibitors implies that cell death is induced as a result of increased intracellular concentrations of a regulatory molecule(s) normally degraded by the ubiquitin/proteasome pathway. A prime candidate for such a regulator of apoptosis appeared to be p53, which is known to be ubiquitinated and degraded by the proteasome (19, 20). Not only is p53 induced by a variety of apoptotic stimuli, but overexpression of p53 has been demonstrated to induce apoptosis in a variety of cell types (4, 5). Consistent with this hypothesis, we found that treatment with proteasome inhibitors resulted in stabilization and rapid accumulation of p53 in both Rat-1 and PC12 cells. Moreover, the accumulated p53 was shown to be biologically active, based on transactivation of the p53 target genes encoding p21 and Mdm2. Finally, we have shown that apoptosis induced by proteasome inhibitors is blocked by expression of dominant-negative p53, and that overexpression of wild-type p53 is sufficient to induce apoptosis of Rat-1 cells in transient transfection assays. Although other molecules may also be involved in cell death, these data suggest that modulation of p53 turnover is a key event in apoptosis induced by proteasome inhibitors.

Our finding that apoptosis is induced by inhibitors of the chymotrypsin-like activity of the proteasome is consistent with a previous report showing that lactacystin, which inhibits all three proteasome activities (22), induces apoptosis of U937 myeloid leukemia cells (23). On the other hand, it has recently been reported that inhibition of the proteasome by either lactacystin or peptide aldehydes prevents apoptosis of thymocytes induced by radiation, glucocorticoids, or phorbol esters and of sympathetic neurons induced by nerve growth factor deprivation (24, 25). In these systems, proteasome activity appears to be required for induction of apoptosis upstream of the ICE family proteases. It appeared possible that this apparent discrepancy was due to the fact that U937 cells and the cells used in the present study were actively proliferating, in contrast to the nonproliferating thymocytes and sympathetic neurons in which proteasome activity was required for induction of apoptosis by other agents. Indeed, Grimm et al. (24) noted that prolonged exposure to proteasome inhibitors actually increased cell death in thymocytes that were not treated with other inducers of apoptosis. Consistent with this possibility, we found that nonproliferating Rat-1 cells were no longer susceptible to apoptosis induced by proteasome inhibitors. However, inhibition of the proteasome still induced apoptosis of nonproliferating PC12 cells that had been induced to differentiate by treatment with nerve growth factor. These results indicate that cell proliferation is one but not the only determinant of cellular response to inhibition of the proteasome. It thus appears that the proteasome can function to promote either cell survival or cell death, depending on both proliferative state and other factors that may be cell type-specific.

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