BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 74–79 (1996)
ARTCILE NO. 0986

Enhancement of CPP32-like Activity in the TNF-Treated U937 Cells by the Proteasome Inhibitors

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Received May 31, 1996

CPP32, which is most closely related to CED-3 in the apoptotic protease in *C. elegance*, is activated during apoptosis induced by anti-Fas and TNF. Since processing of CPP32 is important for the activation, we examined the effects of protease inhibitors on CPP32-like activity in the TNF-treated U937 cells. Unexpectedly, proteasome inhibitors (at 5 μM) such as Z-LLnV, Z-LLL, and lactacystin enhanced CPP32-like activity, Ac-DEVD-MCA degrading activity, in the TNF-treated U937 cells in 3 hr, but E64d, cysteine protease inhibitor, did not. These proteasome inhibitors alone did not enhance CPP32-like activity in the untreated U937 cells under the condition used. The proteasome seems to protect the cells from apoptosis by degrading CPP32-like protease or its processing enzyme.

CPP32, one of the Ced-3/ICE-like proteases, is the mammalian enzyme most closely related to CED-3 (1,2). Poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair and genome surveillance and integrity, is one potential substrate for CPP32, but not ICE, during apoptosis (3-6); PARP cleavage requires a 50-100 fold higher ICE concentration than does processing of an ICE precursor at an equivalent substrate concentration. Inhibition of CPP32-mediated PARP cleavage attenuates apoptosis, demonstrating the importance of this protease in the apoptosis of mammalian cells.

The inactive form of CPP32 is *Mr* 32 K protein (p32), while the active form of CPP32, apopain, is composed of two subunits of *Mr,* 17K (p17) and 12 K (p12) (4). Activated ICE and granzyme B cleave CPP32 onto p17 and p12 *in vitro* (3,7). Recently, it has been shown that CPP32 is cleaved into active form during the Fas-mediated apoptosis (8). Thus CPP32 is activated by the processing during apoptosis.

On the other hand, it has been reported that other proteases than Ced-3/ICE proteases are involved in the Fas-mediated apoptosis of Jurkat cells (9). Inhibitors for calpain have been demonstrated to have inhibitory effects on the activation-induced cell death of T cell hybridomas as well as the death of mature T cells (10,11). Fodrin, a substrate for calpain I, is cleaved during the apoptosis induced by anti-Fas (12). Recently, however, Imajoh-Ohmi *et al.* reported that inhibition of proteasome, a high-molecular-weight intracellular proteolytic enzyme, induces apoptosis of U937 cells (13) and that calpain inhibitors stimulate the apoptosis of U937 cells induced by TNF (14).

In the present study, we examine how the proteasome is involved in the apoptosis induced by TNF. We demonstrate that proteasome inhibitors enhance the degrading activity of Ac-

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Abbreviations: TNF, Tumor Necrosis Factor; ICE, IL-1β Converting Enzyme; MCA, 4-methylcoumaryl-7-amide; CMK, chloromethylketone; LLnL, *N*-acetyl-leucinyl-leucinyl-norleucinal-H; Z-LLnV, carbobenzoxy-leucinyl-leucinyl-norvalinal-H; Z-LLL, carbobenzoxyl-leucinyl-leucinyl-leucinal-H.

0006-291X/96 $18.00

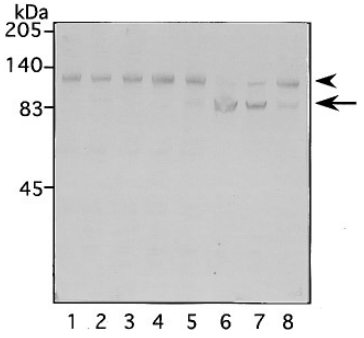

FIG. 1. The effects of proteasome inhibitors on the cleavage of PARP in the TNF treated U937 cells. U937 cells treated with TNF (100 U/ml) in the presence or absence of proteasome inhibitors (10 μM) for 5 hr. Lane 1, untreated cells; lane 2, Z-LLL; lane 3, Z-LLnV; lane 4, lactacystin ; lane 5, TNF; lane 6, TNF and Z-LLL; lane 7, TNF and Z-LLnV; lane 8, TNF and lactacystin. Arrowhead and arrow indicate PARP and 85 kDa fragment, respectively.

DEVD-MCA, synthesis peptide of PARP, as well as the cleavage of PARP, in the TNF-treated U937 cells.

METHODS AND MATERIALS

Detection of cell death. Jurkat cells and U937 cells were cultured in a RPMI-1640 medium containing 10 % FCS in the CO_2 incubator at 37 ℃. Cells were incubated with protease inhibitors in the presence or absence of TNF (50 U/ml) for 4 hr. Cell viability was determined by trypan blue exclusion. Values represent the average viability from three independent wells ($\pm$SD) and are normalized to the percentage of viable cells remaining in the untreated cultures. Two independent experiments showed similar results.

CPP32-like and ICE-like activity. U937 cells and Jurkat cells were incubated with TNF and anti-Fas at various concentrations for indicated periods, respectively. U937 cells were also incubated with protease inhibitors at various concentrations in the presence or absence of TNF (50 U/m) for indicated periods. After incubation, cells were washed two times with PBS, and the cell pellets were lysed in PBS containing 0.2% Triton X 100 on ice for 10 min. After centrifugation at 10,000 $\times$ g for 5 min, the cell extracts (50 μg protein) was incubated with 10 μM Ac-DEVD-MCA (Peptide Institute, Osaka), synthetic peptide of PARP, or Ac-YVAD-MCA (Peptide Institute, Osaka), synthetic peptide of IL-1 β, in the incubation buffer (50 mM Tris-HCl pH7.5, 1 mM DDT) for 20 min in order to measure CPP32-like or ICE-like activities, respectively. The reactions were halted by the addition of 10% SDS. The fluorescence was measured at 380 nm for excitation and at 460 nm for emission.

Immunoblot analysis. U937 cells were incubated with TNF (50 U/ml) in the presence or absence of proteasome inhibitors (10 μM) for 5 hr. After washed two times with PBS, the cell pellets were lysed with the sample buffer (50 mM Tris-HCl pH7.5, 10% glycerol, 1% SDS) and sonicated. After centrifugation at 10,000 $\times$ g for 10 min, the cell extracts (100 μg protein) were subjected to the SDS gel (12%) electrophoresis. Proteins of the gels were electrophoretically transferred to the nitrocellulose filters. After filters were incubated with monoclonal anti-PARP antibody, the reactivities on the filters were detected by alkaline phosphatase-conjugated goat anti-mouse immunogloblin (Promega, Madison, WI) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate.

RESULTS AND DISCUSSION

Anti-Fas and TNF induce the cleavage of PARP, potential substrate for CPP32, during apoptosis (3). The cleavage of PARP in the TNF (50 U/ml)-treated U937 cells was markedly enhanced by proteasome inhibitors (10 μM) such as Z-LLL, Z-LLnV and lactacystin, while the proteasome inhibitors alone did not cleave PARP under the condition used (Figure 1).

To examine the effect of proteasome inhibitors on the cleavage of PARP in more detail, the CPP32-like activity was measured by using the synthetic peptide of PARP as a substrate. The CPP32-like activity was increased in U937 cells by the treatment with TNF (100 U/ml) for 2 hr and in Jurkat cells by the treatment with anti-Fas (100 ng/ml) for 2 hr, whereas ICE-like activity Ac-YVAD-MCA degrading activity, was not increased in 5 hr (Figure 2a). Thus

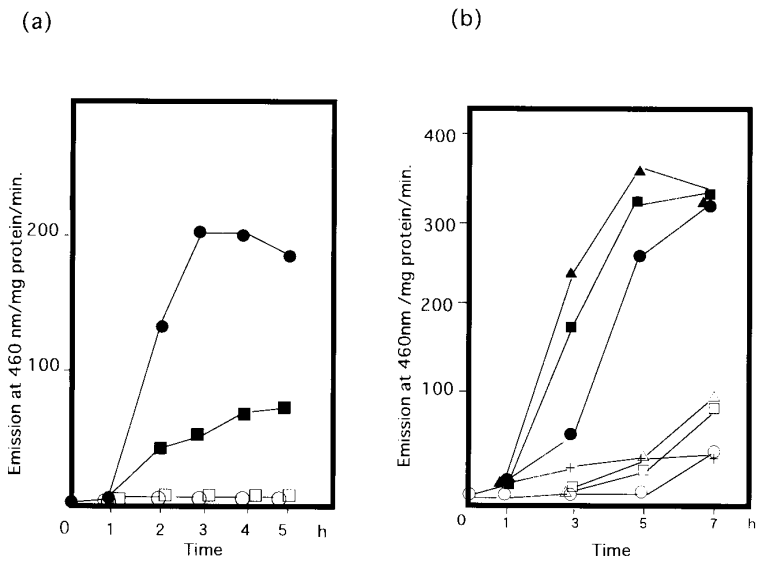

FIG. 2. The CPP32-like activity during apoptosis induced by TNF and proteasome inhibitors. (a) Time-dependent activation of CPP32-like protease in Jurkat cells treated with anti-Fas and U937 cells treated with TNF. CPP32-like and ICE-like activities were detected by measuring the degradation of Ac-DEVD-MCA and Ac-YVAD-MCA, respectively. Circles and squares indicate anti-Fas-treated Jurkat cells and TNF-treated U937 cells, respectively. Closed circles and squares indicate CPP32-like activities and open ones indicate ICE-like activities. (b) Time-dependent effects of the proteasome inhibitors on the CPP32-like activity in the TNF-treated U937 cells. U937 cells were incubated with proteasome inhibitors (5 μM) in the presence or absence of TNF (50 U/ml). Triangles, squares, and circles indicate the cells treated with Z-LLL, Z-LLnV, and lactacystin, respectively. Closed and open ones indicate CPP32-like activities in the presence and absence of TNF, respectively. Crosses indicate the CPP32-like activity in the TNF-treated U937 cells in the absence of proteasome inhibitors.

CPP32-like protease seems to be a key enzyme not only in the Fas-mediated apoptosis but also in the TNF-mediated one.

Z-LLL, Z-LLnV and lactacystin (at 5 μM) increased CPP32-like activity in the TNF (50 U/ml)-treated U937 cells in 3 hr and the CPP32-like activity achieved a maximum level at 5 hr (Figure 2b). Since these proteasome inhibitors alone did not have an ability to activate CPP32-like protease in the untreated U937 cells in 3 hr, but they activated it by the incubation for more than 5 hr, we examined the effect of proteasome inhibitors on the TNF-induced CPP32-like activity by incubating U937 cells with TNF and proteasome inhibitors at various concentrations for 3 hr.

The CPP32-like activity increased dose-dependently in the U937 cells treated with TNF in the presence or absence of proteasome inhibitors and achieved a plateau at 100 U/ml of TNF. (Figure 3a). Z-LLL, Z-LLnV and lactacystin could increase the CPP32-like activity in the U937 cells treated with at least 1 U/ml of TNF for 3 hr, and they could increase the maximum level of CPP32-like activity induced by TNF (100 U/ml) about 6-, 5- and 3-fold, respectively.

The CPP32-like activity in the TNF (50 U/ml)-treated U937 cells was dose-dependently increased by proteasome inhibitors, while ICE-like activity was not increased (Figure 3b). The relative order of efficiency of proteasome inhibitors to enhance CPP32-like activity was Z-LLL, Z-LLnV, LLnL and lactacystin. Z-LLL (1μM), Z-LLnvaH (4 μM), LLnL (8.5 μM) and lactacystin (10 μM) increased the CPP32-like activity in the TNF-treated U937 cells about 4-fold. However, E64d (10 μM), cysteine protease inhibitor, had no effect on the CPP32-like activity. These peptide aldehydes and lactacystin had the similar effects on the CPP32-like activity in the anti-Fas (25 ng/ml)-treated Jurkat cells (unpublished observation).

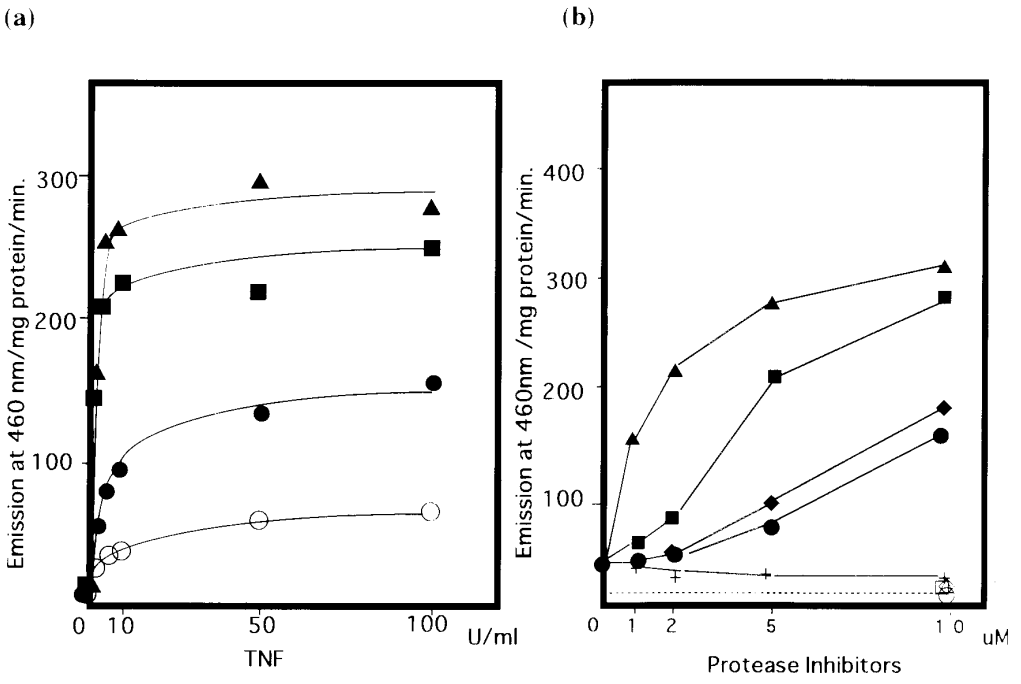

FIG. 3. Effects of the proteasome inhibitors on the CPP32-like activity in the TNF-treated U937 cells (a) Dose-dependent activation of CPP32-like protease in U937 cells by TNF in the presence or absence of proteasome inhibitors. U937 cells were incubated with TNF (1-100 U/ml) in the presence or absence or proteasome inhibitors (5 μM) for 3 hr. Closed triangles, squares and circles indicate the CPP32-like activities in the TNF-treated U937 cells enhanced by Z-LLL, Z-LLnV and lactacystin, respectively. Open circles indicate the CPP32-like activity in the TNF-treated U937 cells. b) Dose-dependent effects of the proteasome inhibitors on the CPP32-like activity in the TNF-treated U937 cells. U937 cells were incubated with TNF (50 U/ml) in the presence of protease inhibitors at various concentrations. Triangles, squares, diamonds, circles and crosses indicate the cells treated with Z-LLL, Z-LLnV, LLnL, lactacystin, and E64d, respectively. Closed ones indicate CPP32-like activities and open ones indicate ICE-like activities. Break line indicates the CPP32-like activity in the untreated U937 cells.

The proteasome plays an essential role in the ATP/ubiquitin-dependent pathway, which is involved in the complete degradation of abnormal and short-lived regulatory proteins (15,16). The 20S proteasome, a molecular weight about 700 kDa, is composed of 13-15 distinct subunits of similar size (21-31 kDa), and contains multiple peptidase activities (15-18). A series of peptide aldehydes, LLnL, Z-LLnV and Z-LLL, have been shown to be potent inhibitors of the chymotryptic site on the 20S proteasome with different dissociation constants (LLnL, 140 nM; Z-LLnV, 21 nM; Z-LLL, 4.0 nM) (19, 20). A less potent inhibitor of the proteasome function, LLnL (20, 21), had only a weak effect on the activation of CPP32-like protease (Figure 3a). The relative order of efficiency of Z-LLL, Z-LLnV and LLnL on the enhancement of CPP32-like activity agrees with their relative abilities to inhibit the proteasome activity. Although these peptide aldehydes also inhibit calpain and lysosomal cathepsin, the three have similar dissociation constants (5-12 nM) against these cysteine proteases. The lysosomal enzymes and calpains are inactivated by E64d, which did not enhance CPP32-like activity in the TNF-treated U937 cells. Moreover, lactacystin, which selectively inhibits proteolytic activities of the proteasome by covalently binding to the amino-terminus of its catalytic β-subunit (22,23), also activated CPP32-like protease (Figure 2a and 3). Consequently, Z-LLL, Z-LLnV and LLnL must be stimulating the CPP32-like activity in the TNF-treated U937 cells by inhibiting of the proteasome function.

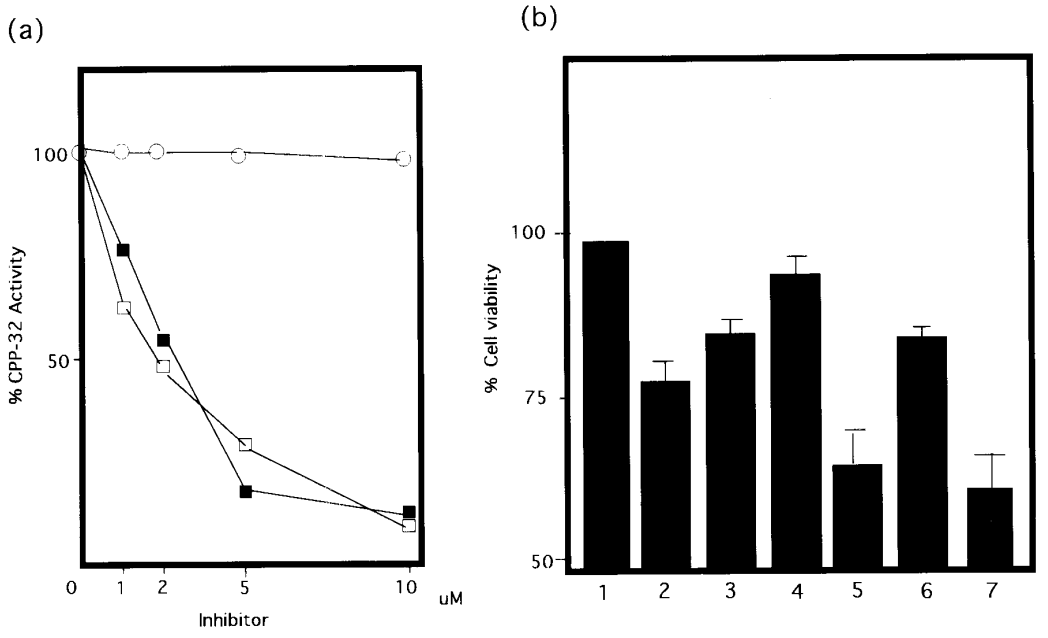

FIG. 4. Effect of Ac-DEVD-CHO and Ac-YVAD-CMK on the CPP32-like activities and apoptosis synergistically induced by TNF and Z-LLnV. (a) Inhibition of CPP32-like activity in U937 cells treated with TNF (50 U/ml) and Z-LLnV (5 μM) by Ac-DEVD-CHO and Ac-YVAD-CMK. U937 cells were incubated with TNF and Z-LLnV in the presence of Ac-DEVD-CHO (open squares), Ac-YVAD-CMK (closed squares) and E64d (open circles) at various concentration for 3 hr. (b) Inhibition of the apoptosis of U937 cells treated with TNF and Z-LLnV by Ac-YVAD-CMK. U937 cells were incubated with TNF (50 U/ml) and Z-LLnV (5 μM) in the presence or absence of Ac-YVAD-CMK (10 μM) for 4 hr. Lane 1, untreated U937 cells; lane 2, TNF; lane 3, TNF and Ac-YVAD-CMK; lane 4, Z-LLnV; lane 5, TNF and Z-LLnV; lane 6; TNF, Z-LLnV and Ac-YVAD-CMK, lane 7; TNF, Z-LLnV and E64d (10 μM).

The CPP32-like activity in the U937 cells treated with TNF (50 U/ml) and LLnV (5 μM) was dose-dependently inhibited by Ac-YVAD-CMK (ICE inhibitor) as well as Ac-DEVD-CHO (CPP32 inhibitor) (Figure 4a). These inhibitors (10 μM) inhibited about 90% of CPP32-like activity, but E64d (10 μM) did not. LLnV enhanced not only the CPP32-like activity in the TNF-treated U937 cells but also the TNF-mediated apoptosis. The TNF and LLnV-mediated apoptosis as well as the TNF-mediated one was inhibited by Ac-YVAD-CMK (10 μM) (Figure 4b) (24,25), suggesting that the activation of YVAD-inhibitable ICE-like protease by TNF is necessary not only for the activation of CPP32-like protease but also for the apoptosis.

It is not yet clear about the molecular mechanisms by which proteasome inhibitors enhanced CPP32-like activity in the TNF-treated U937 cells. One of the possibility is that proteasome inhibitors inhibit the degradation of the processing enzyme of CPP32-like protease. However, since ICE-like protease activity did not increase in the U937 cells treated with TNF and proteasome inhibitors (Figure 3b), it is unlikely that the proteasome degrades ICE-like protease. The other possibility is that proteasome inhibitors inhibit the degradation of CPP32-like protease. Mch-3, a CPP32-like protease, that cleaves PARP has been very recently found, and Mch-3 is processed into active form by the activated CPP32 (26). Since at least two CPP32-like proteases, CPP32 and Mch-3, are expressed in U937 cells (unpublished observation), they may be accumulated in the U937 cells by the treatment with proteasome inhibitors. TNF must be triggering the activation of the CPP32-like protease accumulated in the U937 cells treated with proteasome inhibitors.

Thus the proteasome seems to protect the cells from the apoptosis induced by TNF by degrading the CPP32-like protease or its processing enzyme.

ACKNOWLEDGMENTS

We are grateful to Dr. Guy Poirier, CHUL Research Center for generous gift of monoclonal anti-PARP antibody. This work was supported in part by a grant from the Human Science Foundation for HIV and cell death.

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