

## Participation of a Cathepsin L-Type Protease in the Activation of Caspase-3

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**ABSTRACT.** A previous paper from this laboratory reported the activation of a caspase-3-like protease by a digitonin-treated lysosomal fraction [*FEBS Lett.* 435, 233–236, 1998]. In this study, we examined the effects of specific inhibitors of lysosomal cysteine proteases, such as cathepsins B, S, and L, on the activation of caspase-3 to find out which cathepsin is responsible for the activation. Pro-caspase-3 in the cytosol was cleaved by a lysosomal protease(s) contained in the supernatant of a digitonin-treated crude mitochondrial fraction containing lysosomes (ML) and the cleaved product was detected by Western blotting using anti-caspase-3 antibody. The activation of caspase-3 by the lysosomal protease(s) was pH dependent and the optimum pH for activation was pH 6.6–6.8. This activation was not inhibited by CA-074, a specific inhibitor of cathepsin B, but was strongly inhibited by CLIK-066 and CLIK-181, specific inhibitors of cathepsin L. The inhibitory effect of CLIK-060, a specific inhibitor of cathepsin S, was very weak. Furthermore, the activation of caspase-3 was enhanced by addition of purified cathepsin L only in the presence of the supernatant of the digitonin-treated ML. These results suggested that a cathepsin L-type protease activity might participate in the activation mechanism of caspase-3 in the presence of the supernatant from the ML.

**Key words:** apoptosis/caspase-3/cathepsin L/crude mitochondrial fraction/lysosomal enzyme

It has become accepted that apoptosis or programmed cell death plays an important role in the regulation of tissue development and homeostasis (8, 20). CED-3 protein is an effector of cellular suicide in the nematode and is homologous to the mammalian protein interleukin-1 $\beta$  converting enzyme (ICE, cysteinyl aspartate-specific proteinase-1, caspase-1) and is activated by death signals such as the Fas ligand and

tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (13, 19). Caspase-3 (CPP32 protease) is located in a late step of the protease cascade, and cleaves the “death substrates”.

It was recently reported that cytochrome c (Cyt.c) was involved in the activation of caspase-3 in a cell-free system and that Bcl-2 inhibits the translocation of Cyt.c from mitochondria to the cytosol, thereby blocking the activation of the caspase cascade and the apoptotic process (7, 10). As a molecular mechanism of Cyt.c dependent caspase-3 activation, it was reported that the apoptotic protease activating factor (Apaf) complex (Apaf-1/pro-caspase-9/Cyt.c) leads to caspase-9 activation in the presence of dATP which in turn cleaves and activates caspase-3 (9). Thus, to date two distinct systems that activate caspase-3 have been elucidated. One is a signaling receptor mediated activation of the caspase cascade system and the other is a mitochondrial Cyt.c mediated caspase-3 activation system.

Several investigators have reported that some lysosomal enzymes participate only in a certain type of

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Abbreviations: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; AMC, 7-amino-4-methyl-coumarin; Apaf, apoptotic protease activating factor; Cyt.c, cytochrome c; caspase, cysteinyl aspartate-specific proteinase; DTT, dithiothreitol; ICE, interleukin-1 $\beta$ -converting enzyme; ML, crude mitochondrial fraction containing lysosomes; MPT, mitochondrial permeability transition; PMSF, phenylmethylsulfonyl fluoride; S-100, cytosolic fraction; TNF, tumor necrosis factor.

apoptosis of cells (15, 16). While the molecular mechanism of lysosome-mediated apoptosis remains to be elucidated, we have recently shown that lysosomal cysteine protease(s) activated caspase-3, but not caspase-1 in a cell-free extract (3). In this context, it has been reported that lysosomal cathepsin B activated caspase-11 and caspase-1 (18). These results suggest that there is a lysosomal enzyme-mediated caspase-3 activation system in the signal transduction pathway of apoptosis. Thus it is important to determine what types of lysosomal enzyme(s) participate in the activation of caspase-3.

In this study we examined the effect of specific inhibitors of the various types of cathepsin on the activation of caspase-3 by lysosomal enzymes, and found that cathepsin L-type protease is the most likely candidate of the lysosomal enzymes for the activation of caspase-3.

## Materials and Methods

### Chemicals

Fluorogenic tetrapeptide substrate for acetyl-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA for caspase-1), acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA for caspase-3), acetyl-Val-Glu-Ile-Asp-MCA (Ac-VEID-MCA for caspase-6), and Acetyl-Ile-Thr-Asp-MCA (Ac-IETD-MCA for caspase-8) were obtained from the Peptide Institute (Osaka, Japan). Cyt.c, phenylmethylsulfonyl fluoride (PMSF), aprotinin and pepstatin A were obtained from Sigma Co. Ltd. (St. Louis, MO, USA). dATP was obtained from Amersham Pharmacia Biotech Co. (USA). Pure cathepsins (B and L) were purchased from Cosmo Bio. Co. Ltd. (Tokyo, Japan). Anti-caspase-3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Various inhibitors of cathepsins were kindly donated by the Ohtsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan).

### Preparation of a crude mitochondrial fraction containing lysosomes (ML) and a cytosolic fraction (S-100)

A preliminary ML preparation was isolated from the livers of Wistar rats weighing 200–250 g after overnight fasting. Livers were minced and homogenized with a Potter teflon homogenizer in 10 vol. of 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose. This homogenate was centrifuged at  $750 \times g$  for 10 min at 4°C. The supernatant was collected and centrifuged at  $10,000 \times g$  for 10 min at 4°C to obtain ML, which was washed twice by resuspending it in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose at 4°C and centrifuged again under the same conditions (14). A post mitochondrial fraction of the rat liver homogenate was centrifuged at  $105,000 \times g$  for 60 min and the resultant supernatant

was used as a cytosolic fraction (S-100B). In some experiments, rat liver was homogenized in a medium A (20 mM HEPES buffer (pH 7.5), 1.5 mM  $MgCl_2$ , 1 mM dithiothreitol (DTT), 1 mM EGTA and 1 mM EDTA) containing 0.25 M sucrose for the preparation of a cytosolic fraction (S-100A) (7, 9, 10).

### Activation of caspase-3 in S-100B by lysosomal enzyme(s)

Caspase-3 in the cytosolic fraction (S-100B) was activated by the supernatant of digitonin-treated ML (3). 1.5 mg protein/ml of ML in 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl was treated with 40  $\mu M$  digitonin for 10 min on ice and centrifuged at  $20,000 \times g$  for 10 min. 0.2 ml of this supernatant containing lysosomal enzymes was incubated with 0.1 ml of 1 mg protein/ml of S-100B (pro-caspase-3) at 37°C for 120 min to activate caspase-3.

### Activation of caspase-3 in S-100A by Cyt.c plus dATP

Caspase-3 in the cytosolic fraction (S-100A) was activated by purified Cyt.c plus dATP. 1 mg protein/ml of S-100A was incubated with 10  $\mu M$  Cyt.c and 1 mM dATP at 37°C for 120 min to activate caspase-3.

### Assay for caspase activity

Caspase activity was determined as described previously (3, 22) using synthetic substrates (10  $\mu M$ ) in 20 mM HEPES buffer (pH 7.5) containing 0.1 M NaCl and 5 mM DTT at 37°C for 60 min. Results are presented as picomoles of 7-amino-4-methyl-coumarin (AMC) released per microgram of S-100 protein per minute.

### Assay for cathepsins

Recombinant human cathepsin S was expressed and purified according to the method of Zhao *et al.* (23). Inhibitory properties of cysteine protease by epoxysuccinate derivatives are summarized in Katunuma and Kominami (5). All epoxysuccinate derivatives including CLIks-060, -066 and -181 were synthesized by Katunuma and coworkers according to the methods previously published for E-64 and CA-074 with some modifications (4, 6, 12, 17), and were fully characterized by IR, proton NMR and fast atom bombardment MS.

The inhibitory specificities of these inhibitors were determined using individual pure cathepsins by Katunuma *et al.*. Assay methods for cathepsins followed the principal of Barrett's method with some modifications (1).

### Gel electrophoresis of cytosolic proteins and Western blotting analysis

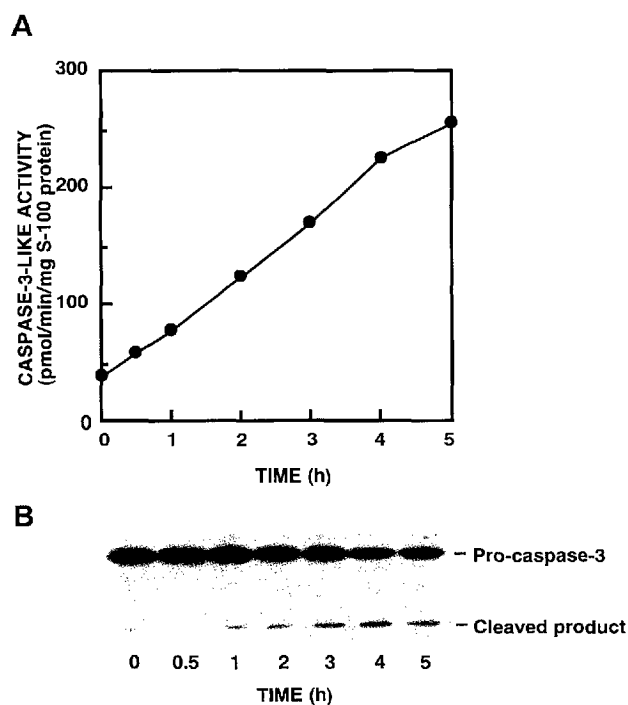
After incubation with the supernatant of a digitonin-treated ML, the S-100 fraction was dissolved in SDS-sample buffer

(125 mM Tris-HCl (pH 6.8), 4% SDS, 1%  $\beta$ -mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and boiled at 100°C for 5 min. Following electrophoresis, proteins were transferred to Immobilon-P filter (Millipore Co.). After blocking with 5% skimmed-powdered milk, caspase-3 was detected on a filter by anti-caspase-3 antibodies and peroxidase-conjugated anti-rabbit IgG. Immunoreactive bands were visualized using an ECL Western blotting detection system (Amersham Co.) (21, 22). Protein concentrations were determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard.

## Results and Discussion

### Detection of a cleaved product of pro-caspase-3 after activation by lysosomal enzyme(s)

To confirm the activation of caspase-3 in S-100B by a digitonin-treated ML, S-100B was incubated with the supernatant from digitonin-treated ML at 37°C for different times and analyzed for caspase-3 activity and a cleaved product of pro-caspase-3 by Western blotting



**Fig. 1.** Time dependent activation of caspase-3 and appearance of a cleaved product of pro-caspase-3 in S-100B after incubation with lysosomal enzymes. Pro-caspase-3 contained in the S-100B fraction (1 mg protein/ml) was incubated with the supernatant of digitonin-treated ML (1.5 mg protein/ml) at 37°C for different times. Caspase-3 activity was measured as described in Materials and Methods and pro-caspase-3 and its cleaved product were analyzed by Western blotting using polyclonal anti-caspase-3 antibody. (A) time dependent activation of caspase-3; (B) time dependent cleavage of pro-caspase-3.

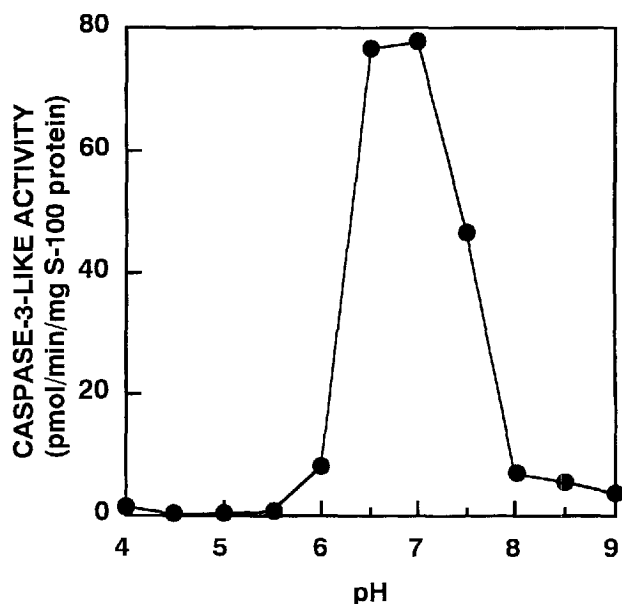
analysis. A cleaved product of caspase-3 increased with the time of incubation in parallel with the increase in caspase-3 activity (Fig. 1). As shown in a previous paper (3), the supernatant of a digitonin-treated ML has large amounts of lysosomal enzymes, such as acid phosphatase, arylsulphatase and *N*-acetyl- $\beta$ -D-glucosaminidase. These experimental results indicated that pro-caspase-3 was cleaved by lysosomal enzymes present in the supernatant of digitonin-treated ML.

### pH dependence for the activation of caspase-3 by lysosomal enzyme(s)

The activation of caspase-3 by a lysosomal enzyme was pH dependent, with an optimum at pH 6.6–6.8 (Fig. 2).

### Effect of specific inhibitors of cathepsin on the activation of caspase-3 by lysosomal enzymes

To determine the molecular species of lysosomal enzyme which activates caspase-3, the effects of specific inhibitors of various cathepsins (12, 17) on the activation of caspase-3 by the lysosomal enzymes were examined. No effect was observed with CA-047, a specific inhibitor of cathepsin B, at any concentration tested (Table I). On the contrary, CLIK-181 and -066, specific inhibitors of cathepsin L, strongly inhibited the activation. CLIK-181 inhibited the activation at any pH tested from pH 6.0 to 8.0 (data not shown). The



**Fig. 2.** pH optimum for the activation of caspase-3 by lysosomal enzymes. Experimental conditions were as in Figure 1 except that the buffer solutions used were 20 mM citrate-NaPi buffer containing 0.15 M KCl for pH 4.0–7.0 and 10 mM Tris-HCl buffer containing 0.15 M KCl for pH 7.5–9.0, respectively.

**Table I.** EFFECT OF VARIOUS INHIBITORS OF CATHEPSINS ON THE ACTIVATION OF CASPASE-3 BY LYSOSOMAL ENZYMES

Inhibitors	Cysteine proteases	Inhibitor concentration (M)				
		10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	10 <sup>-5</sup>	10 <sup>-4</sup>
		% Inhibition				
CLIK-060	Cathepsin L	0	0	30	80	100
	Cathepsin B	0	0	20	70	100
	Cathepsin S	40	80	100	100	100
	Caspase-3 activation	—	0	0	30	80
CLIK-181	Cathepsin L	50	84	100	100	100
	Cathepsin B	0	0	0	4	—
	Cathepsin S	0	0	10	80	100
	Caspase-3 activation	—	20	70	100	100
CLIK-066	Cathepsin L	26	76	100	100	100
	Cathepsin B	0	0	0	0	0
	Cathepsin S	0	0	0	30	—
	Caspase-3 activation	—	40	80	90	90
E-64	Cathepsin L	30	70	90	100	100
	Cathepsin B	40	90	100	100	100
	Cathepsin S	90	100	100	100	100
	Caspase-3 activation	—	30	70	80	90
CA-074	Cathepsin L	0	0	0	10	—
	Cathepsin B	60	100	100	100	100
	Cathepsin S	0	0	0	—	—
	Caspase-3 activation	—	0	0	0	0

Experimental conditions were the same as in Figure 1. The supernatant of digitonin-treated ML (1.5 mg protein/ml) was incubated with S-100B (1 mg protein/ml) in the presence of various concentrations of inhibitors at 37°C for 120 min. Concentrations of inhibitors were 10<sup>-8</sup>–10<sup>-4</sup> M as indicated in the Table. CLIK-060, a specific inhibitor of cathepsin S; CLIK-181 and CLIK-066, specific inhibitors of cathepsin L; E-64, an inhibitor of cysteine protease; CA-074, a specific inhibitor of cathepsin B.

inhibitory activity of CLIK-060, a specific inhibitor of cathepsin S, was not observed within the cathepsin S inhibition range. As shown in Table I, inhibition of cathepsin S by CLIK-060 was observed even at a concentration of 10<sup>-8</sup> M, while inhibition of caspase-3 activation by this compound was observed only at concentrations higher than 10<sup>-5</sup> M, which is in the cathepsin L inhibition range. These results suggested that cathepsin S did not participate in this activation, but that cathepsin L might be involved in the activation of caspase-3. The optimum pH of cathepsin L was at pH 5.0–6.0; however cathepsin L also shows considerable activity at pH 6.5–7.0. Furthermore, the optimum pH changes with different conditions. These results suggested a possible involvement of some unknown protease(s) in addition to cathepsin L in the activation of caspase-3.

#### *Effect of purified cathepsin L on the activation of caspase-3 by lysosomal enzyme*

Of all the lysosomal enzymes, cathepsin L was suggested to be the most likely candidate for the activation of caspase-3. Therefore the effect of purified cathepsin L on the activation of caspase-3 was examined. The activation of caspase-3 was enhanced by adding purified cathepsin L to a reaction mixture consisting of S-100B and the supernatant of the digitonin-treated ML (Fig. 3). However, no effect of cathepsin L was observed in the absence of the supernatant from the digitonin-treated ML. These results indicated that cathepsin L did not directly activate caspase-3. At present, the molecular mechanism of activation is not known but the activation by lysosomal enzymes might be preceded by more than two reaction steps. From this observation, it was suggested that the pH dependency observed in the previous section (Fig. 2) might reflect the combined pH dependency of more than two differ-

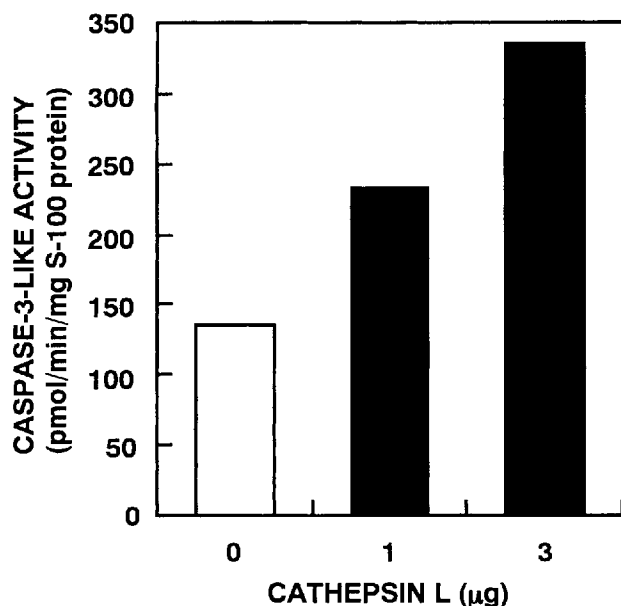


Fig. 3. Effect of purified cathepsin L on the activation of caspase-3 by lysosomal enzymes. Experimental conditions were as for Figure 1. One and 3  $\mu$ g Cathepsin L was added to 0.3 ml of reaction mixture containing the supernatant of digitonin-treated ML and S-100B. 0, without addition; 1 and 3, with addition of 1 and 3  $\mu$ g protein of cathepsin L/0.3 ml.

ent enzymes including cathepsin L.

#### *Effect of various cathepsin inhibitors on the activation of caspase-3 by Cyt.c plus dATP*

As described previously, in many of the published experiments of caspase-3 activation, a cytosolic fraction containing pro-caspase-3 was prepared in the presence of various protease inhibitors in medium A (7, 9, 10). Thus, the inhibitory effects of protease inhibitors on the activation of caspase-3 were not observed. In this experiment, to detect the effects of protease inhibitors on the activation of caspase-3 by Cyt.c plus dATP, we used the S-100A fraction prepared in the absence of any protease inhibitors. The activation of caspase-3 by Cyt.c plus dATP was not inhibited by any of protease inhibitors including the cathepsin inhibitors (Fig. 4).

Preliminary experiments in this laboratory showed that the supernatant of the digitonin-treated ML had no ability to activate caspase-1 and 8 but that it did slightly activate caspase-6. This caspase-6 activation might be due to the activation of caspase-3. However, further experiments were needed to deal with the specificity of caspase-3 activation. In this laboratory it was also shown that some inhibitors of cysteine protease inhibited the apoptosis of HL-60 cells induced by 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), a

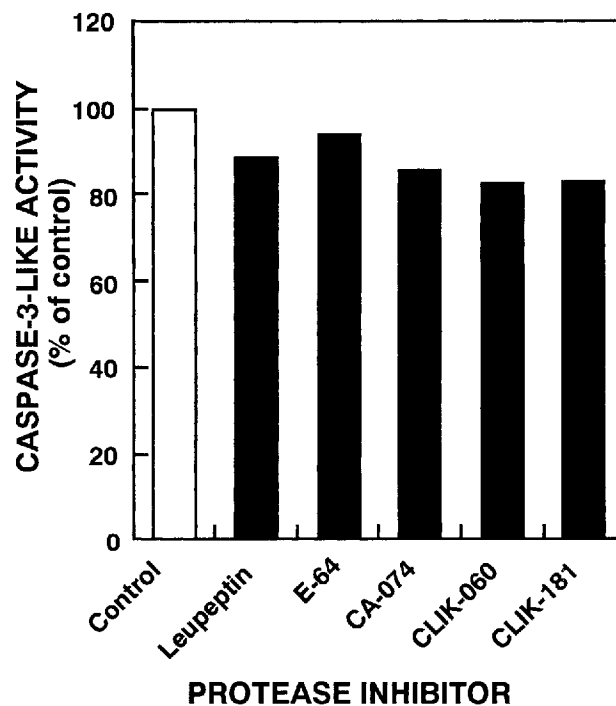


Fig. 4. Effects of various protease inhibitors on the activation of caspase-3 by Cyt.c plus dATP. S-100A was prepared in medium A (20 mM HEPES (pH 7.5), 1.5 mM  $MgCl_2$ , 1 mM DTT, 1 mM EDTA, 1 mM EGTA) containing 0.25 M sucrose. Caspase-3 in S-100A was activated at 37°C for 120 min by 10  $\mu$ M Cyt.c plus 1 mM dATP. Various protease inhibitors were added simultaneously with activators. Concentrations of leupeptin, E-64, CA-074, CLIK-060 and CLIK-181 were 10  $\mu$ g/ml, 10  $\mu$ M, 10  $\mu$ M, 10  $\mu$ M, and 10  $\mu$ M, respectively. Data were expressed as % of control.

radical initiator, and that of Vero cells by verotoxine (unpublished data). These results indicate that some lysosomal enzymes participate in a certain type of apoptosis of cells. These results also support the idea that the system for the activation of caspase-3 by lysosomal enzymes is completely different from that of Cyt.c plus dATP (9) or of caspase-8 (receptor mediated caspase cascade) (2, 13, 19).

To our knowledge this is the first report that a cathepsin L-type protease was involved in the activation mechanism of caspase-3 in the presence of lysosomal fractions.

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