Ceramide induces apoptosis via CPP32 activation

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Abstract Although both ceramide and interleukin-1β converting enzyme (ICE) family proteases are key molecules during apoptosis, their relationship remains to be elucidated. We report here that cell-permeable ceramide induced cleavage and activation of CPP32, a Ced-3/ICE-like protease, but not ICE. Ceramide-induced apoptosis of Jurkat cells was blocked by the CPP32-specific tetrapeptide inhibitor DEVD-CHO, but not by the ICE inhibitor YVAD-CHO. Furthermore, variant Jurkat cells with defective CPP32 activation were resistant to both anti-Fas- and ceramide-induced apoptosis. These results indicate that CPP32 activation is required for ceramide-induced apoptosis, and suggest sphingomyelin-ceramide pathway functions upstream of CPP32.

Key words: CPP32; Ceramide; Apoptosis; Fas; Interleukin-1β converting enzyme

1. Introduction

Apoptosis is a well-regulated process of cell death that is important in events such as embryogenesis, cell growth control and lymphocyte repertoire formation. Recent investigations have revealed that there are several key pathways in apoptotic signal transduction, including the sphingomyelin-ceramide pathway [1,2] and the pathway involving interleukin-1β converting enzyme (ICE)/Ced-3 family proteases [3].

Ceramide is a newly identified lipid second messenger that is generated through the hydrolysis of sphingomyelin by sphingomyelinases (SMase) [1,2]. Signals from cell surface receptors such as Fas (CD95/Apo1) [4–7] and tumor necrosis factor receptor (TNFR) [8–11] activate SMase and generate ceramide, even in the cell-free system [12]. Synthetic cell-permeable ceramide can mimic such apoptosis. This cytotoxic effect is specific because structural analogues such as dihydroceramide do not induce apoptosis [13,14]. These data suggest that ceramide is a physiological mediator of Fas- and TNFR-mediated apoptosis.

ICE family proteases, which are mammalian Ced-3 homologues, have also been implicated in Fas- and TNF-mediated apoptosis [15–18]. However, ICE itself might not be a true mammalian Ced-3 counterpart because apoptosis proceeds almost normally in ICE-deficient mice [19,20]. So far at least seven mammalian Ced-3 homologues have been identified, and all of them are aspartate-directed cysteine proteases capable of inducing apoptosis [21]. Among them, CPP32 (apopain [22], Yama [23]) appears to be a real mammalian Ced-3 counterpart, because it has greater homology (35% identity) with Ced-3 than ICE [24], and has been identified as the enzyme that cleaves poly-ADP ribose polymerase (PARP) which appears to be involved in DNA repair and the maintenance of genome integrity [22,23]. More recently, CPP32 was shown to be the key ICE-like protease in Fas-mediated apoptosis [25].

Although numerous reports have established the role of ceramide and ICE-like proteases in apoptosis, it has not yet been established whether they work independently in separate signal transduction pathways, or one acts upstream of and requires the other. In TNF-induced apoptosis, both ceramide generation [9,10,26] and ICE-like protease activation [17] require TNFRI(p55), especially the death domain, but not TNFRII(p75), raising the possibility that ceramide and ICE-like proteases exist in the same signaling pathway. We therefore addressed this question in the present study and demonstrated that CPP32 acts downstream of ceramide and its activation is required for ceramide-induced apoptosis.

2. Materials and methods

2.1. Reagents and cell lines

N-Acetyl ceramide (C2-ceramide) was purchased from Wako (Osaka), C2-dihydroceramide from Calbiochem and dioctanoylglycerol from Sigma, and dissolved in ethanol. The final ethanol concentration in the culture medium was always less than 0.1%. Mouse IgM anti-human Fas monoclonal antibody (CH-11) was purchased from Medical Biological Laboratories (Nagoya) and anti-CPP32 monoclonal antibody from Transduction Laboratories. Tetrapeptide inhibitors for CPP32 (Ac-DEVD-CHO) and ICE (Ac-YVAD-CHO), and tetrapeptide substrates for CPP32 (Ac-DEVD-AMC) and ICE (Ac-YVAD-AMC) [22,27] were purchased from Peptide Industries (Osaka) and dissolved in DMSO. The human T cell line, Jurkat, was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). An anti-Fas-resistant (FR) subline of Jurkat was established by culturing wild-type Jurkat cells in the presence of 100 ng/ml anti-Fas (CH-11) for 1 month.

2.2. Immunoblotting of CPP32

Jurkat cells (1×10^6) were treated with 10 μM C2-ceramide in RPMI 1640 medium containing 1% FCS for the indicated time periods. Cell pellets were lysed in lysis buffer containing 125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, and 1% β-mercaptoethanol, and boiled for 5 min. The samples were separated on 15% SDS-polyacrylamide gel, electrotransferred to a polyvinylidene difluoride membrane,

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Abbreviations: ICE, interleukin-1β converting enzyme; SMase, sphingomyelinase; TNFR, tumor necrosis factor receptor; PARP, poly-ADP ribose polymerase; FCS, fetal calf serum; AMC, 7-amino-4-methyl-coumarin

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immunoblotted with anti-CPP32 p17 monoclonal antibody, and developed by an enhanced chemiluminescence (ECL) system (Amersham).

2.3. Measurement of protease activity

Cells (7×10^5) were lysed with 300 μl of 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris pH7.5. Aliquots (50 μl) of the extracts were incubated with 40 μM tetrapeptide substrate, 10 mM HEPES, pH7.5, 0.05 M NaCl, and 2.5 mM DTT in 200 μl of reaction mixture for 2 h at 37°C. The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured using an excitation wave length of 365 nm and emission wave length of 450 nm.

2.4. Assessment of apoptosis

Jurkat cells, with or without preincubation with tetrapeptide inhibitors for 3 h, were treated with anti-Fas antibody (200 ng/ml) or C2-ceramide (10 μM) in RPMI 1640 medium containing 1% FCS. At the indicated time points, the cells were harvested and fixed with 1% glutaraldehyde in PBS. After washing with PBS, cells were stained with 0.2 mM Hoechst 33258 and examined by fluorescence microscopy. Nuclei were scored as apoptotic if they exhibited condensation or fragmentation of chromatin. A minimum of 250 cells were counted for quantitation. Cell viability was measured by the WST-1 tetrazolium assay (Dojindo, Kumamoto) [28,29].

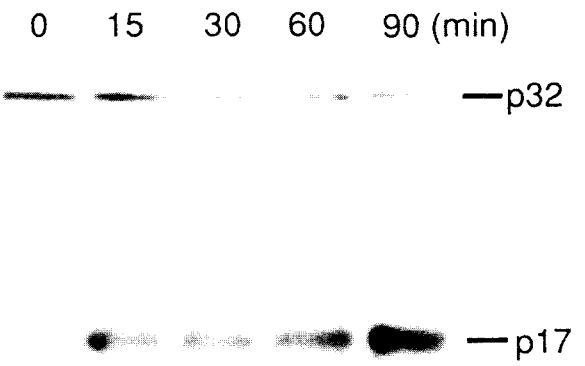

Fig. 1. Cleavage of CPP32 during ceramide-induced apoptosis. The extracts from ceramide-treated cells were subjected to immunoblot analysis using anti-CPP32 p17 antibody. The active subunit (p17) was generated by the cleavage of p32 pro-enzyme in a time-dependent fashion.

3. Results and discussion

In order to examine whether exogenous ceramide induces either CPP32 or ICE activation, we cultured Jurkat cells with cell-permeable C2-ceramide. C2-ceramide induced typical morphological changes seen in apoptosis, such as membrane blebbing, nuclear condensation and fragmentation, and increased numbers of hypodiploid nuclei (not shown). CPP32 is a cysteine protease known to be activated by proteolytic cleavage. p32 precursor was cleaved at two aspartate residues, thus creating two subunits, p17 and p12 [22]. We assessed whether this cleavage occurs during ceramide-induced apoptosis by immunoblotting with anti-p17 antibody. Following the addition of ceramide, the 32 kDa pro-enzyme was cleaved, and the 17 kDa subunit was generated in a time dependent manner (Fig. 1). These results indicated that the proper proteolytic processing of CPP32 occurred during ceramide-induced apoptosis, and suggested that CPP32 was activated. No ICE p45 pro-enzyme was detected in the lysate of Jurkat cells (not shown).

To confirm the CPP32 activation during ceramide-induced apoptosis, cytosolic extracts from the ceramide-treated Jurkat cells were subjected to protease activity assay using tetrapeptide substrate. Enzymatic cleavage after DEVD which is specific to CPP32 [22], was detected as early as 2 h after the addition of ceramide and increased thereafter (Fig. 2). On the other hand, we detected no cleavage after YVAD, which is specific to ICE activity, during ceramide-induced apoptosis (Fig. 2). As recently reported by Schlegel et al. [25], the activity of CPP32 but not ICE was increased during Fas-mediated apoptosis. Its kinetics was similar to that of ceramide-induced apoptosis (Fig. 2). Taken together, the data suggest that CPP32 but not ICE is activated in both ceramide and Fas-mediated apoptosis of Jurkat cells.

The effect of ceramide to induce apoptosis is specific because dihydroceramide, which is a structural analogue of ceramide, does not induce apoptosis [13,14]. We examined the ability of cell-permeable lipids to induce CPP32 activation in Jurkat cells. CPP32 activity induced by C2-dihydroceramide was minimal (Fig. 3), and apoptotic morphological changes were not observed. Dioctanoylglycerol (cell-permeable diacylglycerol) had no effect. The results suggested that the action of ceramide is specific for some target molecules, rather than nonspecific cytotoxic or membrane-toxic effects by cell permeable lipids.

To assess whether CPP32 activation is the essential event in ceramide-induced apoptosis, we determined whether ceramide-induced apoptosis is blocked by CPP32 inhibitor.

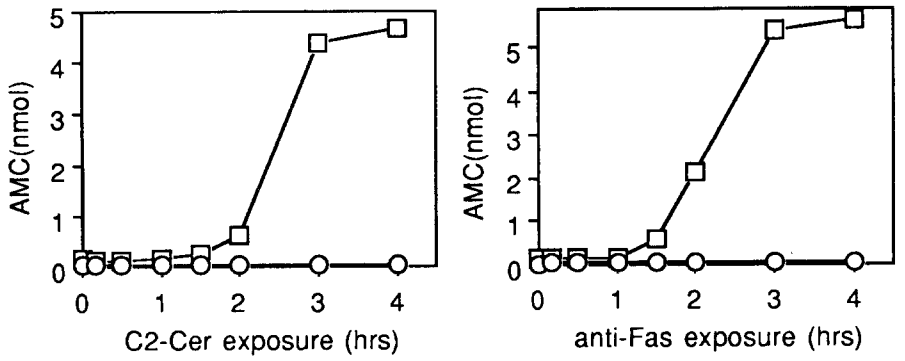

Fig. 2. CPP32 activation induced by ceramide and anti-Fas antibody. Jurkat cells were treated with C2-ceramide (10 μM) or anti-Fas antibody (CH-11, 200 ng/ml) for various incubation periods. Cytoplasmic extracts were examined for protease activity. CPP32 and ICE enzyme activity is expressed as AMC release from the fluorogenic substrates Ac-DEVD-AMC (squares) and Ac-YVAD-AMC (circles), respectively. Data are representative of three experiments.

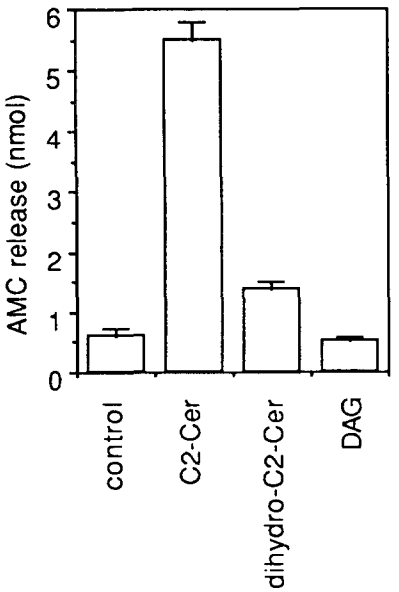

Fig. 3. CPP32 activation induced by cell permeable lipids. Jurkat cells were treated with vehicle alone, 10 μM of C2-ceramide, 10 μM of C2-dihydroceramide or 10 M of DAG (dioctanoylglycerol) for 4 h. CPP32 activity was examined as in Fig. 2. The results are expressed as means ± S.D. of three independent experiments.

CPP32 specific inhibitor, the tetrapeptide aldehyde Ac-DEVD-CHO or ICE-specific inhibitor, Ac-YVAD-CHO [22,27] was added 3 h before ceramide treatment. As shown in Fig. 4A,B, Ac-DEVD-CHO blocked the ceramide-induced apoptosis in a dose-dependent manner. It also inhibited cell morphological changes such as membrane blebbing. In contrast, the ICE inhibitor did not block apoptosis even at a high concentration (Fig. 4A,B). Similarly, Fas-mediated apoptosis was also specifically blocked by the CPP32 inhibitor (Fig. 4B). These results suggested that activation of CPP32, not ICE, is required for both ceramide- and anti-Fas-induced apoptosis.

To further confirm that CPP32 activation is required for ceramide-induced apoptosis, a variant Jurkat cell subline resistant to anti-Fas antibody treatment (Jurkat FR) was studied. It was established by culturing Jurkat cells with 100 ng/ml of CH-11. Although Jurkat FR express the same level of cell surface Fas antigen as wild-type Jurkat (not shown), it was resistant to high doses of CH-11 (Fig. 5A). In this variant, activity of CPP32 after anti-Fas treatment was suppressed (Fig. 5B). Whereas both cell lines expressed a similar level of CPP32 pro-enzyme, cleavage of p32 in Jurkat FR was delayed (not shown). Since the CPP32 inhibitor blocked Fas-mediated apoptosis of Jurkat cells (Fig. 4B), inefficient CPP32 activation appears to be responsible for anti-Fas resistance in Jurkat FR. We therefore examined the sensitivity of this subline to ceramide-induced apoptosis, in order to confirm that it requires CPP32 activation. The results showed Jurkat FR was

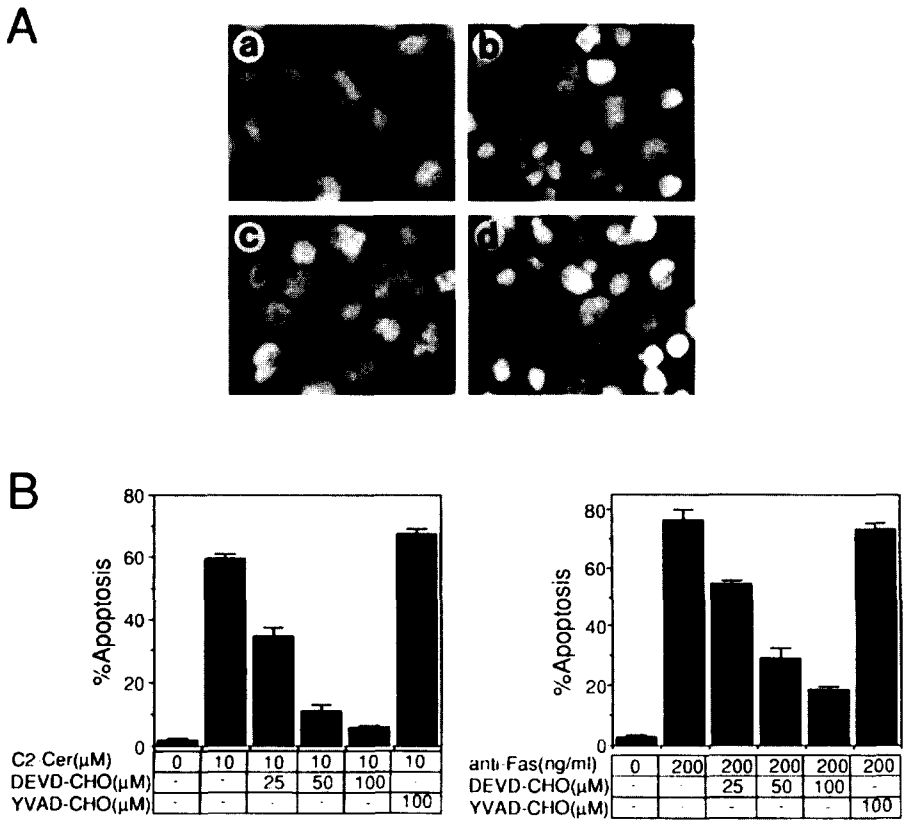

Fig. 4. Specific inhibition of ceramide-induced apoptosis by CPP32 inhibitor. A: Jurkat cells were preincubated with 50 μM CPP32 inhibitor (Ac-DEVD-CHO) (c), 100 μM ICE inhibitor (Ac-YVAD-CHO) (d) or DMSO alone (a,b) for 3 h, then treated with (b–d) or without (a) 10 μM C2-ceramide for an additional 5 h. Cells were fixed with 1% glutaraldehyde and stained with Hoechst 33258. B: Cells were incubated with various doses of CPP32 inhibitor before C2-ceramide or anti-Fas antibody (CH-11) treatment. The percentage of apoptotic cells was determined by examining at least 250 cells.

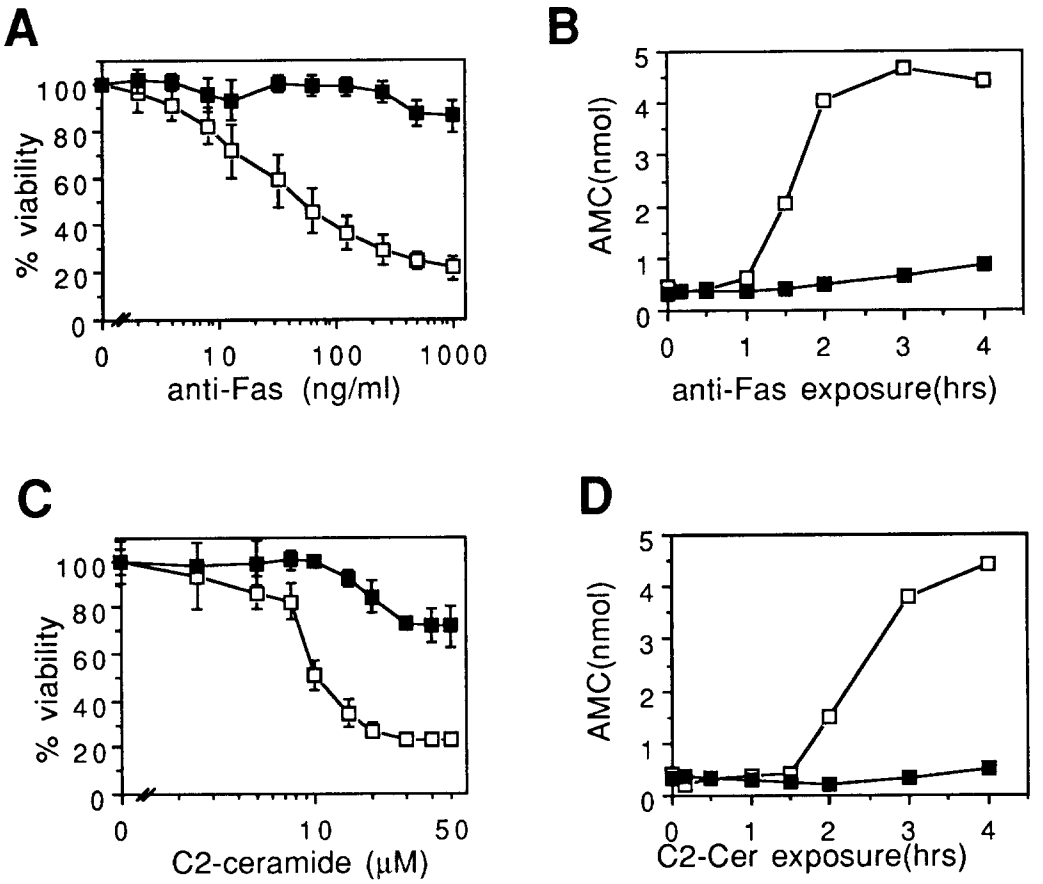

Fig. 5. Resistance to ceramide-induced apoptosis of Jurkat cells with defective CPP32 activation. A,C: Wild-type Jurkat cells (open squares) and anti-Fas-resistant Jurkat cells (Jurkat FR, filled squares) were treated with either CH-11 (A) or C2-ceramide (C) for 5 h. Cell viability was examined by WST-1 tetrazolium assay. B,D: CPP32 enzyme activity of wild-type Jurkat (open squares) and Jurkat FR (filled squares) in response to 200 ng/ml CH-11 (B) and 10 μM C2-ceramide (D).

also more resistant to ceramide-induced apoptosis than wild type Jurkat (Fig. 5C). Indeed, CPP32 activation was also defective during ceramide-treatment (Fig. 5D). The results suggested that Jurkat FR was resistant to ceramide-induced apoptosis because of defective CPP32 activation.

We have demonstrated that ceramide induces apoptosis via CPP32 activation and its activation is required in ceramide-mediated apoptosis of Jurkat cells. This suggests that sphingomyelin-ceramide pathway functions upstream of the ICE family proteases, including CPP32. Recently, Smyth et al. [30] showed that PARP cleavage activity was present during ceramide-induced apoptosis in the Molt-4 T cell line. Since CPP32 activation could be responsible for PARP cleavage activity [22,23], it supports our direct evidence that CPP32 is cleaved and activated during ceramide-induced apoptosis. How ceramide activates CPP32 is unknown. Ceramide itself did not activate CPP32 in vitro (not shown). To date, several molecules have been proposed to be targets for ceramide. They include protein phosphatase 2A (PP2A) [31], 97-kDa serine/threonine protein kinase [32], ζPKC [33] and c-Raf [34]. Ceramide might activate CPP32 through stimulation of these molecules. Further studies are needed to reveal the precise mechanisms between ceramide generation and CPP32 activation.

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