

# CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor

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**DFF45 is a subunit of the DNA fragmentation factor (DFF) that is cleaved by caspase-3 during apoptosis. However, the mechanism by which DFF45 regulates apoptotic cell death remains poorly understood. Here we report the identification and characterization of two mammalian genes, *CIDE-A* and *CIDE-B*, encoding highly related proteins with homology to the N-terminal region of DFF45. *CIDE-A* and *CIDE-B* were found to activate apoptosis in mammalian cells, which was inhibited by DFF45 but not by caspase inhibitors. Expression of *CIDE-A* induced DNA fragmentation in 293T cells, which was inhibited by DFF45, further suggesting that DFF45 inhibits the apoptotic activities of CIDEs. In addition to mammalian *CIDE-A* and *CIDE-B*, we identified DREP-1, a *Drosophila melanogaster* homolog of DFF45 that could inhibit *CIDE-A*-mediated apoptosis. Mutant analysis revealed that the C-terminal region of *CIDE-A* was necessary and sufficient for killing whereas the region with homology to DFF45 located in the N-terminus was required for DFF45 to inhibit *CIDE-A*-induced apoptosis. CD95/Fas-mediated apoptosis was enhanced by CIDEs but inhibited by DFF45. These studies suggest that DFF45 is evolutionarily conserved and implicate CIDEs as DFF45-inhibitable effectors that promote cell death and DNA fragmentation.**

**Keywords:** apoptosis/CAD/cell death/DFF45/DNA fragmentation

## Introduction

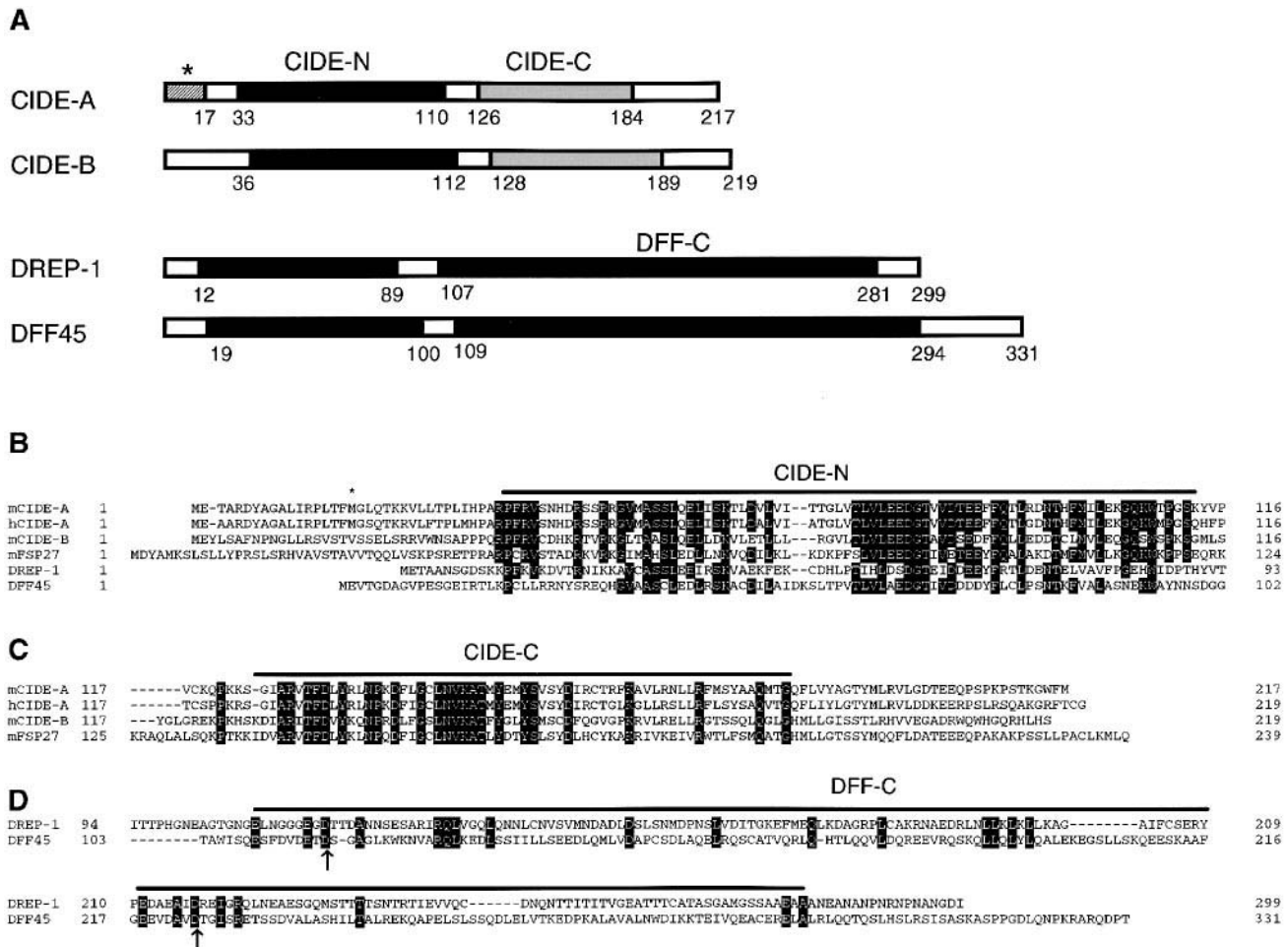
Apoptosis, a morphologically defined form of programmed cell death (PCD) plays an essential role in animal development and tissue homeostasis (Jacobson *et al.*, 1997). The apoptotic mechanism is controlled by an evolutionarily conserved genetic program which is activated in the dying cell (Thompson, 1995; White, 1996; Jacobson *et al.*, 1997). Several regulatory components of the apoptotic pathway have been identified in various living organisms including man (Thompson, 1995; White, 1996). In the nematode *Caenorhabditis elegans*, three genes, *ced-3*, *ced-4* and *ced-9*, play critical roles in the regulation of the death pathway. CED-9, the nematode homolog of Bcl-2 and Bcl-X<sub>L</sub>, binds to CED-4 and represses cell death by interacting with and inhibiting the activity of the cysteine

protease CED-3 through CED-4 (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a,b).

A family of cysteine proteases (designated caspases) related to the *C.elegans* CED-3 protein has been identified in mammalian cells (Alnemri *et al.*, 1996; Cohen, 1997). Caspase activation is induced by a wide array of death signals and leads to cleavage of target proteins and execution of the apoptotic program (Cohen, 1997). Repression of caspase enzymatic activity by viral proteins, p35 from baculovirus and CrmA from cowpox virus, as well as by synthetic peptides inhibits apoptosis induced by stimulation of members of the tumor necrosis factor (TNF) family of cell surface death receptors including CD95/Fas/Apo-1, TNF-R1 and DR3/TRAMP (Nagata, 1997). These observations suggest that the execution phase of the apoptotic process induced by cell death receptors requires caspase activity. However, in other systems of apoptosis, cell death can proceed in spite of caspase inhibition, suggesting the existence of caspase-dependent and caspase-independent mechanisms that are sufficient for cell death (Xiang *et al.*, 1996; McCarthy *et al.*, 1997).

The downstream events that follow caspase activation are poorly understood. Several intracellular substrates including poly(ADP-ribose) polymerase (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995), sterol-regulatory element-binding proteins (SREBPs) (Wang *et al.*, 1995), the U1-associated 70 kDa protein (Casciola-Rosen *et al.*, 1995), the DNA-dependent protein kinase (Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996) and the retinoblastoma B protein (RB) (An and Dou, 1996) are cleaved by activated caspases. However, it is unclear whether the cleavage of any of these proteins plays a pivotal role in the induction of apoptosis.

A heterodimeric protein, designated DNA fragmentation factor (DFF), that mediates DNA fragmentation of isolated nuclei has been purified through cellular fractionation experiments (Liu *et al.*, 1997). DFF is composed of 45 and 40 kDa subunits, of which only the cDNA encoding the 45 kDa subunit has been cloned (Liu *et al.*, 1997). DFF45, but not DFF40, is cleaved by active caspase-3 into three proteolytic fragments, a step that appears to be required for DFF to mediate DNA fragmentation *in vitro* (Liu *et al.*, 1997). However, the effector moiety of DFF that is activated by caspase-3 remains poorly understood. Furthermore, it is unclear whether DFF is necessary for induction of DNA fragmentation and apoptosis *in vivo*. DFF is devoid of nuclease activity when incubated with naked DNA, suggesting that DFF mediates DNA fragmentation by an indirect mechanism perhaps as a component of a signaling cascade that ultimately leads to DNA fragmentation. In order to begin to understand the role of DFF in apoptosis, we searched public databases for novel proteins with homology to DFF45. In the current study, we report the identification and characterization of



**Fig. 1.** Primary structure and alignments of mouse CIDE-A, CIDE-B and FSP27, human CIDE-A and *D.melanogaster* DREP-1. (A) Schematic structure of CIDE-A, CIDE-B, DREP-1 and DFF-45. CIDE-N, CIDE-C and DFF-C domains are shown as closed, light gray and dark gray boxes, respectively. The region specific for CIDE-A\* is shown as a hatched box. (B) Amino acid sequence and alignments of N-terminal regions of CIDEs, FSP27 (mouse), DREP-1 and DFF45. (C) Amino acid sequence and alignments of C-terminal regions of CIDEs and FSP27 (mouse). (D) Amino acid sequence and alignments of C-terminal regions of DREP-1 and DFF45. Proposed conserved blocks are overlined. The putative alternative initial codon of mCIDE-A is indicated by a star. Each entire sequence of mouse CIDE-A, CIDE-B, FSP27, human CIDE-A, DFF45 and *D.melanogaster* DREP-1 is shown in (C) and (D). The nucleotide sequences which encode mouse CIDE-A, CIDE-B and FSP27, human CIDE-A, DFF45 and DREP-1 are available as AF041376, AF041377, M61737, AF041378, U91985 and AF041375, respectively, in GenBank.

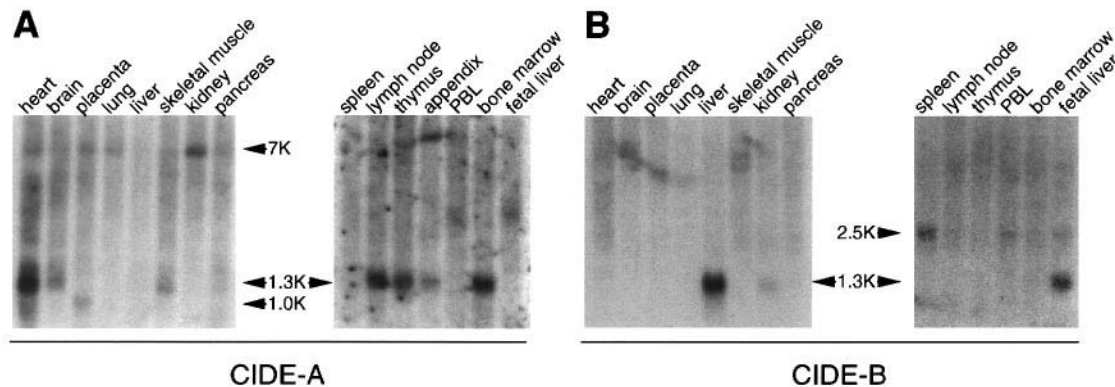
two mammalian genes (*CIDE-A* and *CIDE-B*) and a *Drosophila* gene (*DREP-1*), that encode proteins with significant homology to DFF45.

## Results

### Identification of mammalian CIDEs and *Drosophila* DREP-1

To identify potential *DFF45*-related genes, we searched the expressed sequence tag (EST) database of GenBank for clones with homology to *DFF45* (Liu *et al.*, 1997). Two types of mouse ESTs encoding two distinct peptides with statistically significant amino acid homology to DFF45 were identified ( $P < 2 \times 10^{-3}$ ) by the TBLASTN program. Subsequent sequence analysis revealed that the ESTs represented two different genes with open reading frames encoding highly homologous proteins of 200 and 219 amino acids (Figure 1). We designated these mouse genes *CIDE-A* and *CIDE-B* (for cell death-inducing DFF45-like effector A and B). In addition, a human EST clone with 83% amino acid identity with mouse CIDE-A was identified and it appears, therefore, to represent the

human homolog of CIDE-A (Figure 1). Analysis of the nucleotide sequence of both human and mouse CIDE-A cDNAs revealed two potential in-frame translation initiation sites separated by 51 nucleotides. These two potential initiation codons produce a protein of 217 amino acids (designated CIDE-A\*) and a protein identical to CIDE-A\* but lacking its 17 most N-terminal amino acids (designated CIDE-A). BLAST search revealed that CIDE-A and -B were novel molecules highly homologous to FSP27 (Figure 1B), a protein of unknown function whose expression is associated with terminal differentiation of fat cells (Danesch *et al.*, 1992). The homology of CIDE-A, CIDE-B and FSP27 with DFF45 was restricted to an N-terminal region designated here as CIDE-N domain which showed 39, 29 and 38% amino acid identity respectively with DFF45. Another region of CIDE-A and CIDE-B, termed CIDE-C domain, located in their C-termini shared amino acid homology (54 and 53% identity, respectively) with FSP27 but not with DFF45 (Figure 1). To determine if DFF45-related molecules are conserved in other species, we searched the invertebrate EST database of GenBank for clones with homology to DFF45. We identified a



**Fig. 2.** Tissue distribution of the *CIDE-A* and *CIDE-B* transcripts. Expression of *CIDE-A* (A) and *CIDE-B* (B) in human tissues was analyzed by Northern blot analysis. PBL, peripheral blood leukocytes. X-ray films were exposed for 16 h.

*Drosophila melanogaster* gene encoding a peptide with significant homology to DFF45, designated here as DREP-1. The similarity between DREP-1 and DFF45, CIDE-A and CIDE-B was most significant at the N-termini. Significantly, the C-terminus of DREP-1 has homology to that of DFF45 but not to that of CIDE-A, CIDE-B and FSP27 (Figure 1B and D). Importantly, two aspartic acid residues known to be part of a caspase-3 recognition site in DFF45 (Liu *et al.*, 1997) were also conserved in *Drosophila* DREP-1 (Figure 1D).

#### Differential expression of *CIDE-A* and *-B* mRNA in human tissues

We performed Northern blot analysis to determine the distribution of CIDE-A and CIDE-B RNA transcripts in various human tissues. Expression of CIDE-A was detected in heart and at a lower level in skeletal muscle, brain, lymph node, thymus, appendix and bone marrow as a 1.3 kb transcript. A smaller transcript of 1.0 kb was expressed at low levels in the placenta. In addition, another transcript of ~7.0 kb was detected with the CIDE-A probe in kidney and at a lower level in heart, brain, placenta and lung (Figure 2A). The expression pattern of CIDE-B was different from that of CIDE-A. Hybridization with a CIDE-B probe revealed a major transcript of 1.3 kb in adult and fetal liver (Figure 2B). In addition, another transcript of ~2.5 kb was detected at lower levels in spleen, peripheral blood lymphocytes, bone marrow and fetal liver (Figure 2B). CIDE-A but not CIDE-B mRNA was expressed in 293T embryonic kidney, MCF-7 breast carcinoma and SHEP neuroblastoma cells (data not shown).

#### *CIDE-A* and *-B* but not DFF45 activate apoptotic cell death

To begin to elucidate the physiological function of CIDE proteins, expression constructs producing CIDE-A, CIDE-B and DFF45 were introduced into 293T cells and subsequently observed for features of apoptosis. The CIDE-A-, but not DFF45-transfected cells, displayed morphological features of adherent cells undergoing apoptosis, such as becoming rounded with plasma membrane blebbing, condensed nuclei and detachment from the dish (data not shown). In addition, CIDE-A, but not DFF45, induced nuclear condensation and fragmentation, a feature

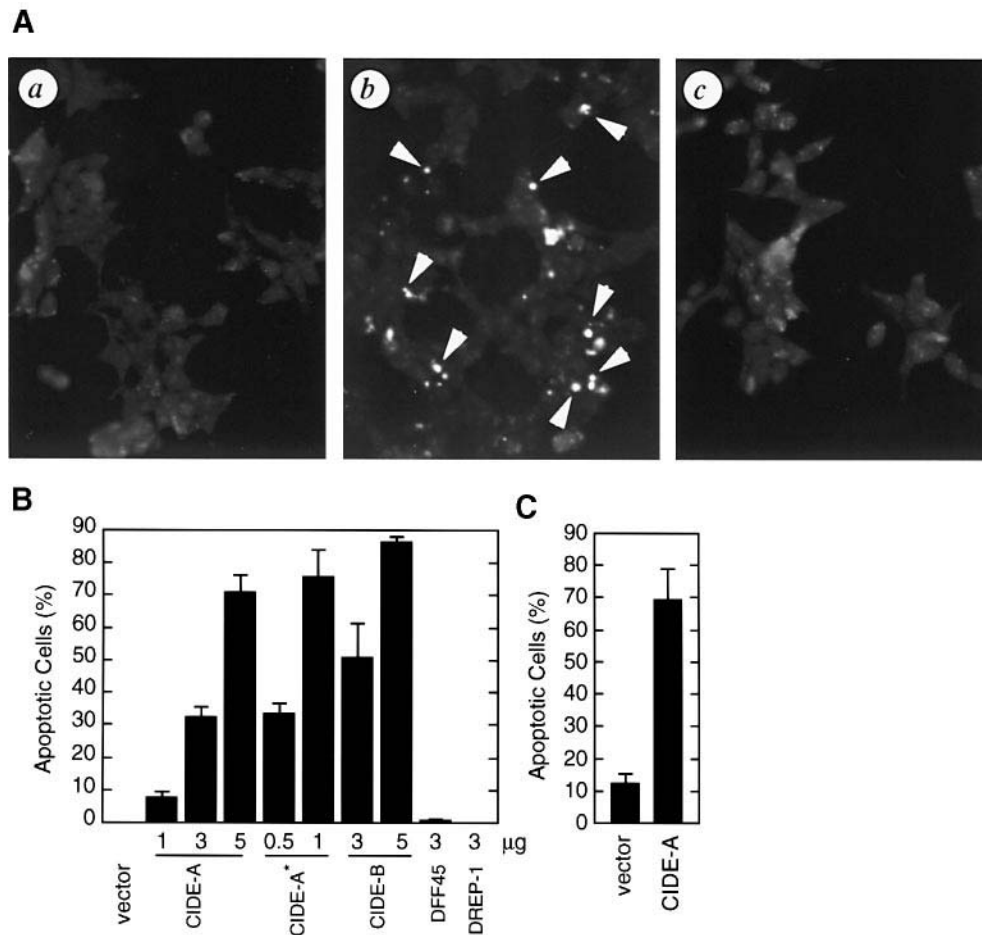
characteristic of apoptosis (Figure 3A). Expression of both CIDE-A and CIDE-A\*, the products of the two potential initiation codons, induced significant killing of 293T cells in a dose-dependent manner as did CIDE-B (Figure 3B). In contrast, expression of DFF45 or the related protein DREP-1 did not induce apoptosis (Figure 3B). In addition to 293T cells, expression of CIDE-A induced significant apoptotic cell death in MCF-7 cells as compared with control plasmid (Figure 3C,  $P < 0.001$ ). In addition, CIDE-A and CIDE-B induced apoptosis in SHEP cells (data not shown). We confirmed these results in cells transfected with plasmids expressing untagged CIDE-A\*, CIDE-A, CIDE-B, DREP-1 and DFF45, indicating that the untagged and Flag-tagged proteins exhibit the same activities when transfected in cells (data not shown). Western blot analysis revealed that CIDE-A, CIDE-A\*, CIDE-B, DREP-1 and DFF45 were expressed in cells, indicating that the lack of expression did not account for the inability of DFF45 to induce apoptosis (data not shown, and Figure 7). These results indicate that CIDE-A and CIDE-B proteins, but not DFF45, induce apoptosis in mammalian cells. Thus, CIDE-A and CIDE-B exhibit a non-overlapping pattern of expression in human tissues.

#### *CIDE-A* and *-B*-induced apoptosis is inhibited by DFF45 and *Drosophila* DREP-1

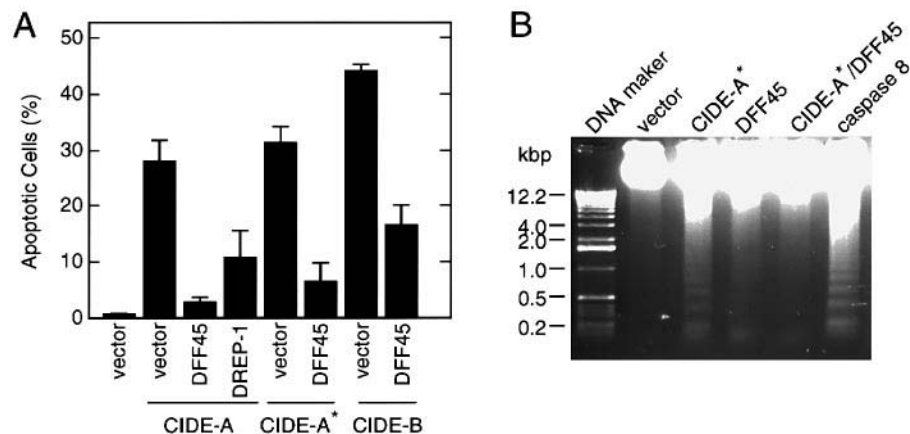
To determine if DFF45 modulates the ability of CIDEs to induce cell death, these proteins were co-expressed in 293T cells and the level of apoptosis was measured. Expression of DFF45 significantly inhibited the killing activity of CIDE-A and CIDE-B (Figure 4A,  $P < 0.001$ ). Furthermore, the *Drosophila* DREP-1 protein also suppressed the ability of CIDE-A to induce apoptosis, further suggesting that DREP-1 is a homolog of DFF45 (Figure 4A,  $P < 0.001$ ).

#### Expression of *CIDE-A* induces oligonucleosomal DNA fragmentation that is inhibited by DFF45 in 293T cells

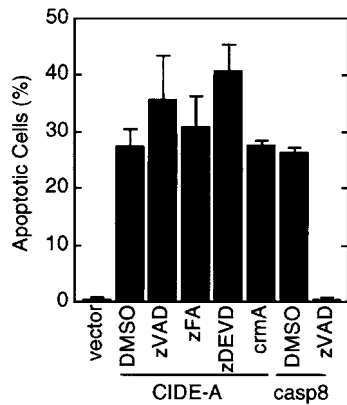
Activation of DFF induces DNA fragmentation of isolated nuclei *in vitro* (Liu *et al.*, 1997). Therefore, we tested next whether expression of CIDE-A or DFF45 could induce DNA fragmentation in 293T cells. Consistent with the results presented in Figure 4A, CIDE-A and caspase-8 (positive control) promoted the fragmentation of genomic



**Fig. 3.** CIDEs induce apoptosis in mammalian cells. (A) Ectopic expression of CIDE-A induces apoptosis of 293T cells. 293T cells ( $3 \times 10^5$ ) were co-transfected with 2  $\mu$ g of pcDNA3 (a), pcDNA3-Flag-CIDE-A (b) or pcDNA3-Flag-DFF45 (c). Nuclei of transfected cells were stained at 18 h after transfection as described (Inohara *et al.*, 1997b). Magnification  $\times 400$ . (B) Apoptosis of 293T cells was induced by CIDE-A and CIDE-B but not by DFF45 and DREP-1. 293T cells ( $3 \times 10^5$ ) were co-transfected with the indicated amount of expression plasmid of Flag-tagged CIDE-A, CIDE-A\*, CIDE-B, DFF45 or DREP-1 together with a  $\beta$ -galactosidase-expressing reporter construct. The data (mean  $\pm$  SD) represent the percentage of round, apoptotic cells with membrane blebbing as a function of total  $\beta$ -galactosidase-positive cells ( $n = 3$ ). (C) Ectopic expression of CIDE-A induces apoptosis in MCF7 cells. MCF7 cells ( $1 \times 10^5$ ) were co-transfected with pcDNA3 or pcDNA3-Flag-CIDE-A and a  $\beta$ -galactosidase-expressing reporter construct. The percentage of apoptotic cells was determined as in (B).



**Fig. 4.** DFF45/DREP-1 can inhibit apoptosis induced by CIDEs. (A) DFF45 can block apoptosis induced by CIDE-A and CIDE-B. 293T cells ( $3 \times 10^5$ ) were co-transfected with pcDNA3 vector alone, 2  $\mu$ g of pcDNA3-Flag-CIDE-A, 0.5  $\mu$ g of pcDNA3-Flag-CIDE-A\* or 2  $\mu$ g of pcDNA3-Flag-CIDE-B plus pcDNA3 or 3  $\mu$ g plus pcDNA3 or pcDNA3-Flag-DFF45. The total amount of DNA was always 5  $\mu$ g, adjusted with pcDNA3. The percentage of apoptotic cells was determined as in Figure 3. (B) DFF45 can inhibit DNA fragmentation induced by CIDE-A\*. 293T cells ( $3 \times 10^5$ ) were co-transfected with pcDNA3 vector alone or with 1  $\mu$ g of pcDNA3-Flag-CIDE-A\*, 3  $\mu$ g of pcDNA3-Flag-DFF45, 1  $\mu$ g of pcDNA3-Flag-CIDE-A\* plus 3  $\mu$ g of pcDNA3-Flag-DFF45 or 1  $\mu$ g of pcDNA3-caspase-8-AU1. The total amount of DNA for transfection was always 5  $\mu$ g, adjusted with pcDNA3. The genomic DNA was extracted from cells and aliquots of one-tenth were loaded into agarose gel.



**Fig. 5.** Caspase inhibitors cannot block CIDE-A-induced apoptosis. 293T cells ( $3 \times 10^5$ ) were co-transfected with pcDNA3 vector alone, 2  $\mu$ g of pcDNA3, 2  $\mu$ g of pcDNA3-Flag-CIDE-A, 2  $\mu$ g of pcDNA3-Flag-CIDE-A plus 2  $\mu$ g of pcDNA3-crmA or 1  $\mu$ g of pcDNA3-caspase-8-AU1. At 8 h after transfection, 20  $\mu$ M of the caspase inhibitor, zVAD-fmk or zDEVD-fmk, or a control peptide, zFA-fmk dissolved in dimethylsulfoxide (DMSO) was added. No cell death was observed 8 h after transfection. Cell death assays were performed as in Figure 3B.

DNA into oligonucleosomal fragments (Figure 4B). Moreover, DNA fragmentation induced by CIDE-A was inhibited by DFF45 (Figure 4B).

#### **Apoptosis induced by CIDE-A is unaffected by caspase inhibitors zVAD-fmk, zDEVD-fmk or the cowpox protein CrmA**

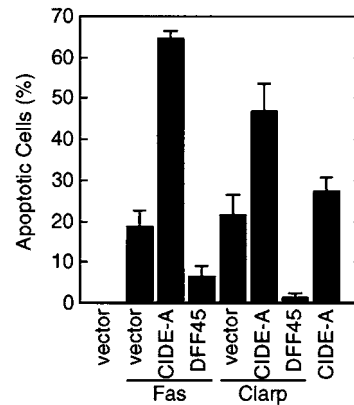
We next determined whether apoptosis activated by CIDE-A expression is caspase dependent. In these experiments, 293T cells were transfected with constructs producing CIDE-A in the presence or absence of caspase inhibitors and the level of apoptosis in the cells was evaluated. The killing activity of CIDE-A was unaffected by the broad-spectrum caspase peptide inhibitors zVAD-fmk and zDEVD-fmk or CrmA, a product of the cowpox virus that inhibits caspases (Figure 5). In control experiments, apoptosis induced by caspase-8 was inhibited by the same concentration of zVAD-fmk (Figure 5).

#### **Apoptosis mediated by CD95/Fas is partially inhibited by DFF45**

An important pathway that transmits signals leading to cell death in mammals is that activated through the CD95/Fas receptor (Nagata, 1997). We next tested whether CIDE-A and DFF45 could modulate apoptosis induced by CD95/Fas signaling. Figure 6 shows that expression of CIDE-A enhanced the apoptotic activities induced by Fas signaling and CLARP, a caspase-like protein (also called CASPER/cFlip<sub>L</sub>/I-FLICE/FLAME-1/CASH) that interacts with caspase-8, a component of the CD95/Fas pathway (Inohara *et al.*, 1997a). In contrast, DFF45 inhibited apoptosis induced by Fas and CLARP (Figure 6).

#### **The C-terminal region of CIDE-A is necessary and sufficient for killing whereas its N-terminus is required for DFF45 to inhibit CIDE-A-induced apoptosis**

We engineered two mutant forms of CIDE-A to characterize further the ability of CIDE-A to induce apoptosis. Mutant CIDE-A (1–107) contains the N-terminal half of



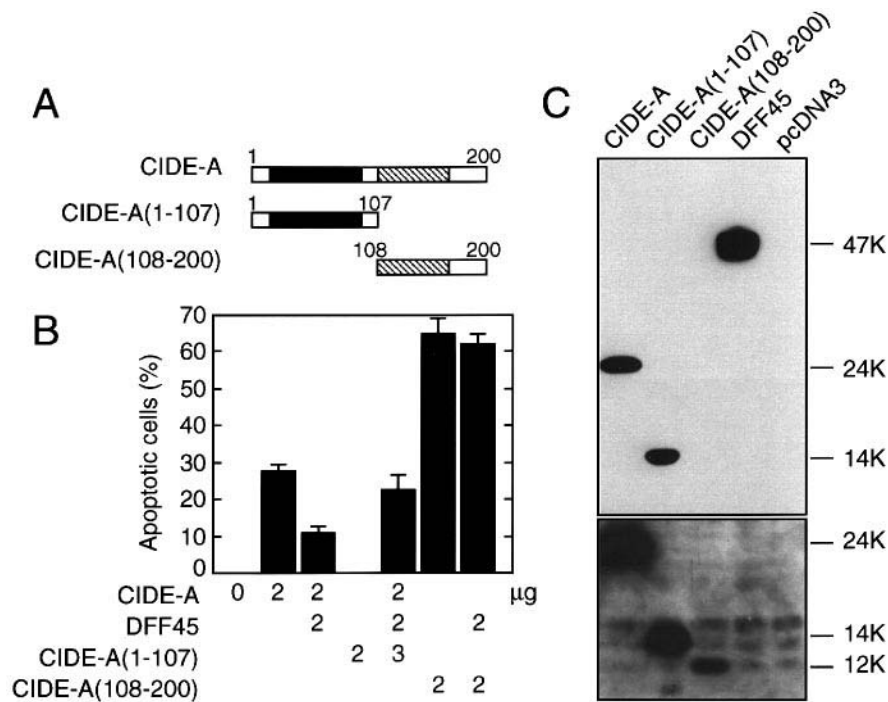
**Fig. 6.** Fas-, CLARP-induced apoptosis is enhanced by CIDE-A and blocked by DFF45. 293T cells ( $3 \times 10^5$ ) were co-transfected with 2  $\mu$ g of CIDE-A or DFF45 expression plasmids plus 0.7  $\mu$ g of pcDNA3-Fas or 1  $\mu$ g of pcDNA3-CLARP-HA. Cell death assays were performed as in Figure 3B.

the protein with homology to DFF45, whereas mutant CIDE-A (108–200) contains the C-terminal half (Figure 7A). CIDE-A (108–200) was capable of inducing apoptosis, whereas the CIDE-A (1–107) mutant did not (Figure 7B), indicating that the killing activity of CIDE-A resides in its C-terminal half. While apoptosis induced by wild-type CIDE-A was inhibited by DFF45 (Figure 4A and B), apoptosis of the CIDE-A (108–199) mutant was not inhibited by DFF45 (Figure 7B), implying that the N-terminal region was required for the inhibition by DFF45. Significantly, the killing activity of the CIDE-A mutant (108–200) was greater than that elicited by wild-type CIDE-A (Figure 7B), suggesting that the CIDE-A activity could be regulated negatively by endogenous proteins such as DFF45 through its N-terminal region (residues 1–107). Consistent with the latter, expression of the mutant CIDE-A (1–107) protein antagonized the inhibition of CIDE-A-induced apoptosis by DFF45 (Figure 7B). Immunoblot analysis revealed that wild-type CIDE-A and mutant CIDE-A (1–107) proteins were expressed at comparable levels whereas the expression of the CIDE-A (108–200) mutant that exhibited killing activity was detected at lower levels (Figure 7C, lower panel), perhaps due to the high level of apoptosis induced by the latter mutant form of CIDE-A (Figure 7B). These results indicate that the C-terminus of CIDE-A is necessary and sufficient for killing while the N-terminal region with homology to DFF45 is required for DFF45 to inhibit CIDE-A-mediated apoptosis.

## **Discussion**

We describe here CIDE-A and CIDE-B, two members of a family of proteins that activate apoptosis in mammalian cells. Another member of the family is FSP27, a protein associated with terminal differentiation of fat cells (Danesch *et al.*, 1997). The expression of FSP27 is regulated by the tumor necrosis pathway, but it is not known if FSP27 is involved in the regulation of cell death (Williams *et al.*, 1992). CIDEs and FSP27 contain an N-terminal region with homology to DFF45, a subunit of DFF (Liu *et al.*, 1997).

CIDE-A and CIDE-B activate apoptosis and appear to



**Fig. 7.** Mutation analysis of CIDE-A. (A) Scheme of CIDE-A mutants. (B) The CIDE-C domain induced apoptosis but not CIDE-N, which is required for inhibition of CIDE-A-induced apoptosis by DFF45. A total of  $3 \times 10^5$  293T cells were co-transfected with 2 μg of pcDNA3-Flag-CIDE-A, pcDNA3-Flag-CIDE-A (1–107), pcDNA3-Flag-CIDE-A (108–200), pcDNA3-Flag-DFF45 or vector control. At 24 h after transfection, Flag-tagged proteins were detected with monoclonal anti-Flag antibody. Cell death assays were performed as in Figure 3B. (C) Expression of CIDE-A, its mutants and DFF45. 293T cells were transfected as above and lysed with NP-40-containing buffer at 24 h after transfection. Tagged proteins were detected with an ECL kit with X-ray film. Images exposed for 10 s and 30 min are shown in the upper and lower panels, respectively. A total of 100 μg of protein was loaded in each lane.

function as positive effectors of the apoptotic pathway. Recent studies have provided insight into the effector mechanism that mediates DNA fragmentation and clues as to how CIDEs could function in apoptosis. Two proteins, CAD and ICAD, have been identified as downstream regulators of DNA fragmentation (Enari *et al.*, 1998; Sakahira *et al.*, 1998). ICAD exhibits high homology to DFF45 and appears to represent its mouse ortholog (Enari *et al.*, 1998). ICAD interacts with and inhibits the activity of CAD, a 40 kDa protein that exhibits DNase activity and probably represents the mouse counterpart of DFF40 (Enari *et al.*, 1998). Apoptotic stimuli that activate caspase-3 can cleave ICAD/DFF45, leading to the release and activation of CAD (Enari *et al.*, 1998), a hypothesis that is compatible with the finding that caspase-3 is required for activation of DFF (Liu *et al.*, 1997). CIDEs could function as signaling components that regulate the ability of CAD to mediate DNA fragmentation. In this model, CIDEs act as upstream regulators of CAD or alternatively they could be co-factors of CAD required for DNase activity. In this regard, CAD has been shown to exhibit DNase activity when expressed in mammalian cells or in an *in vitro* reticulocyte system in the presence of ICAD and caspase-3 (Enari *et al.*, 1998). It is possible that additional factors in the cell lysates may have contributed to the DNase activity. The observation that DNA fragmentation activity induced by CIDEs was not affected by caspase inhibitors is consistent with a model in which CIDEs and CAD act in concert downstream from caspases to mediate DNA fragmentation. However, we cannot rule out an alternative model in which CIDEs could be part of

a DFF45-inhibitable pathway that leads to the activation of a DNase activity distinct from CAD.

The C-terminal region of CIDEs appears to contain the effector domain since this region was necessary and sufficient for killing activity. The N-terminal region of CIDEs, which is homologous to that of DFF45, could regulate the killing activity of CIDEs perhaps through homophilic interactions. These could involve association of CIDEs with DFF45 or with other proteins containing domains with homology to the N-terminal region of DFF45. This hypothesis is supported by these observations. First, the region of CIDEs with homology to DFF45 was required for DFF45 to inhibit CIDE-mediated apoptosis. Second, the killing activity of the CIDE-A mutant (108–200) was greater than that of the wild-type protein, suggesting that CIDE-A is regulated negatively through its N-terminal region that has homology to DFF45. Finally, a mutant form of CIDE-A (1–107) that contained the region with homology to DFF45 antagonized the DFF45 inhibition of CIDE-A-induced apoptosis. However, we have been unable to show that DFF45 and CIDEs interact in mammalian cells (N. Inohara and G. Núñez, unpublished observations), suggesting that proteins other than DFF45 could be involved in these interactions. A possible candidate is CAD. Future experiments will determine whether CIDEs interact with and regulate the activity of CAD.

We have observed that CIDEs induce DNA fragmentation as well as other morphological features of apoptosis, including nuclear condensation and membrane blebbing. Two possibilities can be envisioned to explain these observations. First, DNA cleavage elicited by CIDEs could

result in secondary activation of effectors responsible for morphological features of apoptosis observed upon CIDE expression. Alternatively, CIDEs could directly engage cytosolic and/or nuclear targets in addition to those responsible for DNA fragmentation.

We have identified DREP-1, a *Drosophila* protein related to DFF45. DREP-1 protein may represent the fly homolog of DFF45/ICAD. Three observations support this notion. First, DFF45/ICAD and DREP-1 share significant amino acid and structural homology. The similarity between DREP-1 and DFF45/ICAD was most significant at the N-termini, but also extended to their C-terminal regions. Second, two aspartic acid residues known to be part of a caspase-3 recognition site in DFF45/ICAD were also conserved in DREP-1. These two aspartic residues in DFF45/ICAD are cleaved during the activation of DFF by caspase-3 (Liu *et al.*, 1997) and are important for the release of ICAD from CAD (Enari *et al.*, 1998; Sakahira *et al.*, 1998). Finally, both DFF45 and DREP-1 can inhibit apoptosis induced by CIDEs. However, these results only suggest that DREP-1 is the *Drosophila* ortholog of DFF45/ICAD, and additional experiments are required to establish a role for DREP-1 in programmed cell death. The conservation of DFF45/ICAD in flies suggests an important role for these proteins in both vertebrate and invertebrate apoptosis.

## Materials and methods

### Isolation of the DFF45-related genes

cDNA clones of mouse CIDE-A, human CIDE-A, mouse CIDE-B and DREP-1 were found in the GenBank EST database, using the TBLASTN program. The EST clones 337992 (mCIDE-A), 351557 (mCIDE-A and mCIDE-A\*), 152917 (hCIDE-A), 551880, 790532 (mCIDE-B), LD15946, LD16627 (DREP-1) and 525788 (hDFF45), which encode whole proteins, were obtained from IMAGE Consortium, and their nucleotide sequences were determined by dideoxy sequencing.

### Northern blot analysis

The entire cDNA inserts of EST clones 337992 (mCIDE-A) and 790532 (mCIDE-B) were radiolabeled by random priming using a commercial kit (Boehringer Mannheim) and applied for analysis of human poly(A)<sup>+</sup> RNA blots from various tissues (Clontech Laboratories) according to the manufacturer's instructions.

### Construction of expression plasmids

The entire cDNA inserts of EST clones 337992 (mCIDE-A), 351557 (mCIDE-A\*), 790532 (mCIDE-B), LD15946 (DREP-1) and 525788 (hDFF45) were cloned into the *EcoRI* and *NotI* sites of pcDNA3 (Invitrogen) to produce expression plasmids. To produce tagged proteins, we constructed pcDNA3-Flag and pcDNA3-HA which are derivatives of pcDNA3 (Invitrogen) and share common restriction enzyme sites for cloning. The entire open reading frame of mCIDE-A, mCIDE-A\*, mCIDE-B, DREP-1 and hDFF45 were inserted into the *XbaI* and *Apal* sites of pcDNA3-Flag to produce N-terminal Flag-tagged proteins. Deletion mutants CIDE-A (1–107) and CIDE-A (108–200) were constructed by a two-step PCR mutagenesis method as described (Inohara *et al.*, 1997b). pcDNA3-crmA and pcDNA3-caspase-8-AU1 were described previously (Inohara *et al.*, 1997a). The human CLARP was cloned into the *BamHI* and *XhoI* sites of pcDNA3-HA to generate C-terminal HA-tagged CLARP protein. The authenticity of all constructs was confirmed by dideoxy sequencing and the expression of tagged proteins was confirmed by Western blot using monoclonal antibodies to Flag (Kodak) and HA (Boehringer Mannheim).

### Transfection, expression and immunodetection of tagged proteins

A total of  $5 \times 10^6$  human 293T cells (Numa *et al.*, 1995) were transfected with expression plasmids by a calcium phosphate method as described (Inohara *et al.*, 1997a). The total amount of transfected plasmid DNA

was adjusted with pcDNA3 plasmid to be always the same within individual experiments. After transfection, 293T cells were harvested at 24 h and lysed with 0.2% NP-40 isotonic lysis buffer (Inohara *et al.*, 1997b). Total lysates were subjected to 12% SDS-PAGE and immunoblotted with monoclonal antibodies to Flag (Kodak).

### Apoptosis assays, fluorescence staining of nuclear DNA and DNA fragmentation assay

A total of  $5 \times 10^5$  293T cells were co-transfected with 0.2  $\mu$ g of pcDNA3- $\beta$ -gal plus each expression plasmid in triplicate by the calcium phosphate method as reported (Inohara *et al.*, 1997a). MCF7 were co-transfected with vector control or CIDE-A and a  $\beta$ -galactosidase-expressing reporter construct by Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. In some experiments, 20  $\mu$ M zVAD-fmk [benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone], zFA-fmk [benzyloxycarbonyl-Phe-Ala-(OMe)-fluoromethylketone] and zDEVD-fmk [benzyloxycarbonyl-Asp-Glu-Val-Asp-(OMe)-fluoromethylketone] were added to the medium at 8 h after transfection. zFA-fmk, zVAD-fmk and zDEVD-fmk were obtained from Enzyme Systems. At 24 h after transfection, cells were fixed, stained for  $\beta$ -galactosidase as described (Inohara *et al.*, 1997a) and assayed for morphological features of apoptosis. At least 300 blue-staining cells were counted. Statistical significance was determined by one-way ANOVA followed by Student-Neuman-Keuls post-hoc comparisons.

Nuclear staining with acridine orange was performed as described (Inohara *et al.*, 1997b). Genomic DNA was extracted and analyzed for DNA fragmentation as described (Liu *et al.*, 1997).

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