Molecular cloning and characterization of CIDE-3, a novel member of the cell-death-inducing DNA-fragmentation-factor (DFF45)-like effector family

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DNA fragmentation is one of the critical steps in apoptosis, which is induced by DNA fragmentation factor (DFF). DFF is composed of two subunits, a 40 kDa caspase-activated nuclease (DFF40) and a 45 kDa inhibitor (DFF45). Recently a novel family of cell-death-inducing DFF45-like effectors (CIDEs) has been identified. Among CIDEs, two from human (CIDE-A and CIDE-B) and three from mouse (CIDE-A, CIDE-B and FSP27) have been reported. In this study human CIDE-3, a novel member of CIDEs, was identified upon sequence analysis of a previously unidentified cDNA that encoded a protein of 238 amino acids. It was shown to be a human homologue of mouse FSP27, and shared homology with the CIDE-N and CIDE-C domains of CIDEs. Apoptosis-inducing activity was clearly shown by DNA-fragmentation assay of the nuclear DNA of CIDE-3 transfected 293T cells. The expression pattern of CIDE-3 was different from that of CIDE-B. As shown by Northern-blot analysis, CIDE-3 was expressed mainly in human small intestine, heart, colon and stomach, while CIDE-B showed strong expression in liver and small intestine and at a lower level in colon, kidney and spleen. Green-fluorescent-protein-tagged CIDE-3 was revealed in some cytosolic corpuscles. Alternative splicing of the CIDE-3 gene was also identified by reverse transcription PCR, revealing that two transcripts, CIDE-3 and CIDE-3α, were present in HepG2 and A375 cells. CIDE-3 comprised a full-length open reading frame with 238 amino acids; in CIDE-3α exon 3 was deleted and it encoded a protein of 164 amino acids. Interestingly the CIDE-3α isoform still kept the apoptosis-inducing activity and showed the same pattern of subcellular localization as CIDE-3. Consistent with its chromosome localization at 3p25, a region associated with high frequency loss of heterozygosity in many tumours, CIDE-3 may play an important role in prevention of tumorigenesis.

Key words: alternative splicing, caspase, cell death, DNA fragmentation, nucleosomal units.

INTRODUCTION

Apoptosis is an evolutionarily conserved phenomenon that plays an important role in the regulation of normal cellular activities in eukaryotes [1]. It is also a critical factor in the pathogenesis of a variety of diseases including: cancers, neurodegenerative disorders, autoimmune diseases and viral infections [2,3]. Apoptotic cells undergo a cascade of events that are characterized by distinct morphological changes, such as membrane blebbing, cytoplasmic and nuclear condensation, the aggregation of chromatin, and the formation of apoptotic bodies [4,5]. The biochemical hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal units, which appears to be the final stage of cell death [6–8].

The fragmentation of DNA associated with apoptosis is induced by the DNA fragmentation factor (DFF) [9]. DFF is composed of two subunits, a 40 kDa caspase-activated nuclease (DFF40) and a 45 kDa inhibitor (DFF45) [9,10]. A novel family of cell-death-inducing DFF45-like effectors (CIDEs) has been identified. They share the conserved amino acid sequence similar to the N-terminal domains in DFF45, as well as in DFF40 [11,12]. Within the CIDEs family, two members, CIDE-A and CIDE-B, have been identified in human and three members, CIDE-A, CIDE-B and FSP27, have been identified in mouse. Besides the conserved N-terminal sequence within all members of the CIDEs family, there exists also a C-terminal conserved sequence important for their function. The N-terminal CIDE-N of the CIDE-B domain performed a weak-interaction interface, which functions as a regulatory domain, and the C-terminal CIDE-C domain was reported to be necessary for the mitochondrial localization and dimerization that are required for the induction of apoptosis [13,14].

We were interested to see whether a FSP27 gene homologue existed in human. The present study describes the molecular cloning and functional characterization of CIDE-3 and its isoform CIDE-3α, a novel member of the CIDEs family. This protein is a human homologue of mouse FSP27 and can induce cell apoptosis. CIDE-3 is located on chromosome 3p25, a region which is deleted in many tumour tissues with high frequency and might be important to the pathogenesis of various cancers.

MATERIALS AND METHODS

Samples of human tissues and cell lines

The samples of human adipose and breast tissues were obtained from Zhongshan Hospital (Shanghai, China). Informed consent was obtained from the patients. The cell lines COS-1, 293T, HepG2, HeLa, A375, Tca8113 and MCF-7 were obtained from

Abbreviations used: CIDE, cell-death-inducing DFF45-like effector; DFF, DNA fragmentation factor [comprising a 40 kDa caspase-activated nuclease (DFF40) and a 45 kDa inhibitor (DFF45)]; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; LOH, loss of heterozygosity; ORF, open reading frame; RT-PCR, reverse transcription PCR; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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The data for the CIDE-3 cDNA sequence have been deposited in the GenBank Nucleotide Sequence Database under the accession number AF303893.

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the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Cloning of cDNA for CIDE-3

We designed primers P1, 5′-GCTGACAAGGATGGAATACG-3′ and P2, 5′-CTTGTGGGCACTACCAGTTAA-3′ by reference to the nucleotide sequence of the cDNA, which was determined with the GrailEXP program [15]. An 803 bp fragment was amplified from a SuperScript® human liver cDNA library (Gibco BRL, Grand Land, NY, U.S.A.) by PCR and sequenced after subcloning into the pMD 18-T vector with a TA cloning kit (Takara, Dalian, China). A 1175 bp fragment was obtained with primer P1 and T7 promoter primer (5′-TAATACGACTCACTATAGGGAGA-3′) by 'touch-down' PCR [15a] from the cDNA library (the first cycle was 94 °C for 30 s, with a melting temperature of 65 °C for 30 s and extension at 72 °C for 90 s. The melting temperature was decreased by 0.5 °C in every subsequent cycle until it was reduced to 50 °C, followed by an additional 15 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s). This PCR product included the 3′-terminal sequence of the cDNA. Owing to the low abundance of CIDE-3′ cDNA in the library used, the 5′-terminal sequence was obtained with primer P3, 5′-AGAATGTTCTTTTGGCCACTGT-3′ and primer P2, by reverse transcription (RT)-PCR from human malignant melanoma cell line A375.

Total RNA was extracted with TRIzol® reagent (Gibco BRL), in accordance with the manufacture's instructions, from human adipose tissue, breast tissue, and cell lines. Aliquots (3 μg) of total RNA were reverse transcribed with SuperScript® II RNase H^- reverse transcriptase (Gibco BRL). The reaction mixture was subjected to PCR with primers P1 and P2. The PCR products were separated by electrophoresis on a 1.5 % agarose gel. Two fragments were obtained by RT-PCR and the fragments obtained from the breast tissue sample were sequenced.

Northern-blot analysis

The 803 bp PCR product was radiolabelled by random priming with a commercial kit (Takara, Osaka, Japan) and used for Northern-blot analysis of human poly(A)$^+$ RNA blots from various tissues (obtained from Professor Minyue Zhang, Nanjing University, China) in Rapid-hyb buffer (Amersham, Buckinghamshire, U.K.). Hybridizations were performed according to the manufacturer's instruction. Northern-blot analysis of transcripts encoding CIDE-B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed similarly with probes obtained from the SuperScript® human liver cDNA library.

Construction of expression plasmids

The entire open reading frame (ORF) of CIDE-3 cDNA was cloned into the *Xho*I and *Xba*I sites of pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) to produce an expression plasmid. The entire ORFs of CIDE-B, CIDE-3 and CIDE-3α were inserted into the *Xba*I and *Eco*RI sites of pcDNA3-FLAG (kindly provided by Dr Gabriel Nunez, University of Michigan Medical School, Michigan, U.S.A.) for production of N-terminal FLAG-tagged proteins. The entire ORF of CIDE-3 was inserted into the *Eco*RI and *Sal*I sites of pEGFP-C2 (Clontech, Palo Alto, CA, U.S.A.) for production of N-terminal green fluorescent protein (GFP)-tagged protein. The entire ORF of CIDE-3α was inserted into the *Xho*I and *Eco*RI sites of pEGFP-C3 (Clontech) for production of N-terminal GFP-tagged protein.

Transfection, Western-blot analysis and DNA-fragmentation assay

We seeded 9×10^5 human embryonic 293T cells in 60 mm dishes, 24 h prior to transfection. Cells were transfected using Lipofectamine™ (Gibco BRL) according to the manufacturer's instructions. A total of 5 μg of plasmids DNA and 12.5 μl of Lipofectamine™ were added to cells in each 60 mm dish. Cells were harvested 24 h after transfection. For Western blotting, one-sixth of the cells from a dish were washed once with PBS and lysed with 50 μl of lysis buffer A [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 % Triton X-100, 1 mM PMSF, 10 mM dithiothreitol, 5 μg/ml aprotinin]. The cell lysate was incubated for 10 min on ice and mixed with 50 μl of 2 × loading buffer for SDS/PAGE. A 10 μl aliquot of the mixture was then subjected to SDS/PAGE (15 % gel). Proteins were transferred to a PVDF membrane (NEN, Boston, MA, U.S.A.) and immunoblotted with Anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO, U.S.A.). Proteins were detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA, U.S.A.) and visualized with ECL® reagent (Perfect, Shanghai, China). The remainder of the cells from individual dishes were washed once with PBS and lysed in 500 μl of lysis buffer B [5 mM Tris/HCl (pH 8.0), 20 mM EDTA, 0.5 % Triton X-100]. Each cell lysate was incubated for 10 min on ice and extracted with phenol and chloroform (1:1, v/v) and then with chloroform and 3-methylbutan-3-ol (24:1, v/v). The DNA was precipitated with 0.1 vol. of 5 M NaCl and 2.0 vol. of 100 % ethanol for 1 h on ice. The pellet was dried and resuspended in TE buffer (pH 8.0) after centrifugation. The sample was treated with RNase A for 1 h at 37 °C prior to electrophoresis on a 1.5 % agarose gel. Ethidium bromide-stained DNA was visualized with UV light and photographed.

Assessment of apoptosis and fluorescence staining of nuclear DNA

We co-transfected 3×10^5 CHO cells with 0.25 μg of pcDNA3-β-gal, plus 1.5 μg of each expression plasmid, using 3.5 μl of Lipofectamine™. Then, 24 h after transfection, cells were fixed in a mixture of 4 % paraformaldehyde in PBS for 20 min at 21 °C and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Takara) at 37 °C for 3 h. At least 300 blue-stained cells were examined for morphological features of apoptosis, as described elsewhere [14], after each transfection ($n = 3$) in random fields under a phase-contrast microscope. Nuclei were stained with Acridine Orange and ethidium bromide as described previously [16].

Subcellular localization

We transfected 3×10^5 COS-1 cells with 2 μg of plasmids encoding GFP-CIDE-B (kindly provided by Dr Peng Li, Institute of Molecular and Cell Biology, Singapore), CIDE-3 or CIDE-3α using Lipofectamine™. Then, 23 h after transfection, cells were incubated with Mitotracker Red CMXRos (Molecular Probes, Eugene, OR, U.S.A.), as described previously [14]. Cells were mounted on slides for examination under a Leica SP2 confocal microscope.

RESULTS

Cloning of a cDNA for human CIDE-3

To identify a novel member of the CIDEs family in human, we searched the high-throughput genomic sequences of GenBank. One genomic clone AC007783, including several sequences that encoded potential peptides with statistically significant homology

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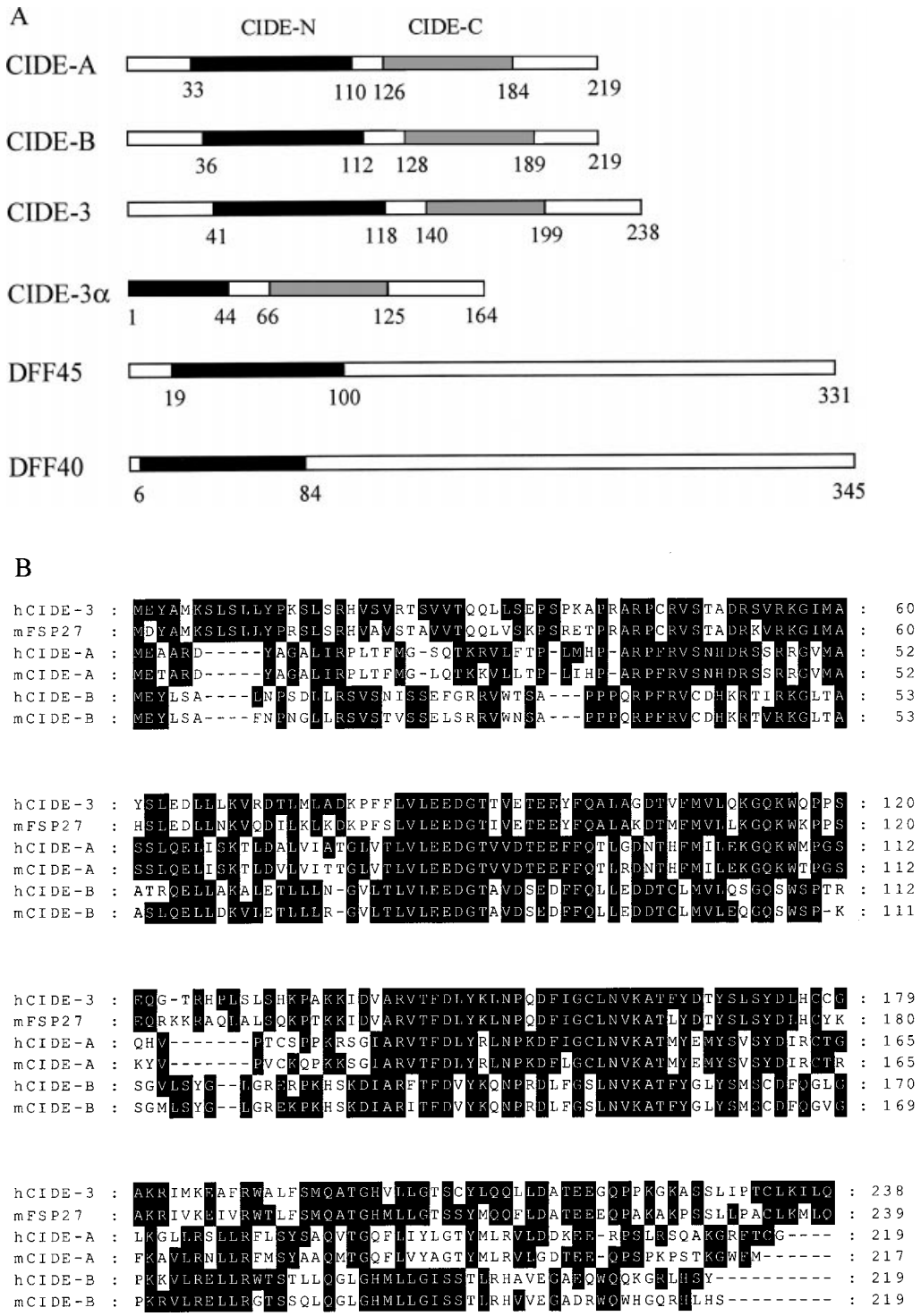

Figure 1 Primary structures and alignment of members of the CIDEs family

(**A**) Schematic structure of human protein family: CIDE-A, CIDE-B, CIDE-3, CIDE-3α, DFF45 and DFF40. The CIDE-N and CIDE-C domains are shown as black and grey boxes, respectively. The numbers indicate the amino acid residues of the putative CIDEs protein family. (**B**) Deduced amino acid sequences and alignment of six members of the CIDEs family. The shaded regions indicate identical amino acid residues. The positions of the amino acid residues are listed on the right.

to CIDE-B, was found. By using the GrailEXP program [15], it was determined that this clone comprised the ORFs encoding a homologous protein to CIDE-B. We designed primers P1 and P2 (see the Materials and methods section) on the basis of our observations and obtained an amplified product from a human cDNA library by PCR. Sequence analysis revealed that this product encoded a novel protein of 238 amino acids. To obtain the 3′-terminal sequence of this cDNA, we performed touch-

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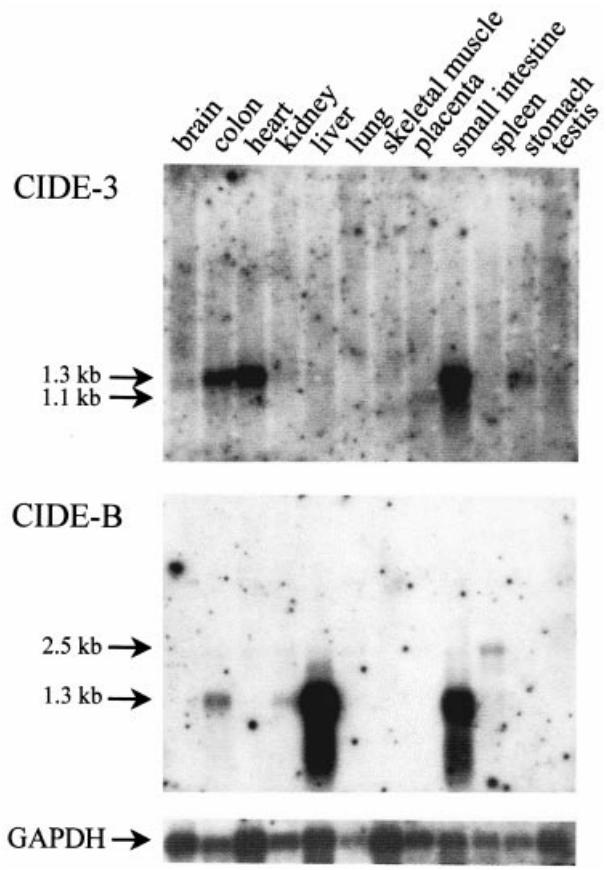

Figure 2 Distribution of CIDE-3 and CIDE-B transcripts

The expression of CIDE-3 (upper panel) and CIDE-B (middle panel) transcripts in human tissues was analysed by Northern-blot analysis with an [α-^{32}P]CTP random primed DNA fragment of the cDNA for CIDE-3 or CIDE-B respectively or with a cDNA probe for GAPDH as a control (lower panel).

down PCR [15a] with primer P1 and T7 promoter primer. Owning to the low abundance of CIDE-3 cDNA in the library used, the 5′-terminal sequence was obtained by RT-PCR from human malignant melanoma cell line A375. The cDNA consisted of 1305 nt with an ORF that encoded 238 amino acids. An ATG triplet, starting at nt 141, matches the consensus sequence of translation initiation codons (RNNATGG) compiled by Kozak [17]. The putative polypeptide had a predicted molecular mass of 26.8 kDa. The deduced amino acid sequence of the cDNA product was strongly homologous (66%) with that of mouse FSP27, which is a protein of unknown function whose expression is associated with the terminal differentiation of fat cells [18]. Moreover, the protein was moderately homologous with human CIDE-A (44%) and CIDE-B (38%) at the amino acid level (Figure 1). The amino acid sequence and the results of the functional analysis described below, led us to designate this protein 'cell death-inducing DFF45-like effector 3' or 'CIDE-3'.

Tissue distribution of CIDE-3 transcripts

We performed Northern-blot analysis to determine the distribution of CIDE-3 transcripts in various human tissues. The 1.3 kb CIDE-3 transcript was expressed mainly in the small intestine, heart, colon and stomach and at a lower level in brain, kidney and liver. A small transcript of 1.1 kb was detected at a lower level in placenta. In contrast, the CIDE-B transcript of 1.3 kb was expressed at a higher level in liver and small intestine and at a lower level in colon and kidney. A longer CIDE-B transcript of 2.5 kb was detected at a lower level in spleen (Figure 2). The expression pattern of the gene for CIDE-3 was different from that of gene for CIDE-B.

Expression and subcellular localization of GFP-tagged CIDE-B and CIDE-3

To investigate the subcelluar localization of CIDE-3, the sequence encoding the GFP was fused, in-frame, to the N-terminal of CIDE-3, and the resultant plasmids were transiently transfected into COS-1 cells. Overexpressed GFP-tagged CIDE-3 was presented in a punctuated manner in the cytosol (Figure 3). CIDE-3 displayed in an aggregated form near some mitochondria. Staining of CIDE-3 with Golgi-, endoplasmic reticulum- or lysosomes-specific markers showed no overlapping staining (results not shown). The exact subcellular localization of CIDE-3 remains to be clarified. In contrast to previously published results suggesting that CIDE-B protein was localized in mitochondria [14], we did not observe a specific staining pattern for mitochondria with a probe for CIDE-B. CIDE-B protein was present in some cytosolic corpuscles and the corpuscles that CIDE-B formed were smaller than those of CIDE-3.

Induction of apoptotic cell death by CIDE-3 and CIDE-B

To elucidate the physiological function of CIDE-3 protein, we introduced expression constructs that encoded CIDE-B and CIDE-3 into 293T cells and then examined the cells for features of apoptosis. The transfected cells developed the morphological features of adherent cells that are undergoing apoptosis, becoming rounded with blebbing of the plasma membrane, condensed nuclei and detachment from the dish. CIDE-3 induced both the condensation of nuclei (Figure 4A) and the fragmentation of DNA (Figure 4B), two characteristic features of apoptosis, as did CIDE-B. We confirmed these results with cells transfected with plasmids that encoded non-tagged CIDE-3. The non-tagged and FLAG-tagged proteins had the same activities when expressed in transfected cells (results not shown). Western-blot analysis confirmed that CIDE-B and CIDE-3 had been expressed in the cells (Figure 4C). The extent of CIDE-3-induced apoptosis of CHO cells was dose-dependent, as was the case with CIDE-B (Table 1).

Identification of alternative splicing of the CIDE-3 gene and cloning of an isoform, CIDE-3α

Two different sizes (1.1 and 1.3 kb) of CIDE-3 gene transcript were produced, as indicated by the expression pattern in different tissues on Figure 2 (upper panel). Therefore, we tested whether alternative splicing of the CIDE-3 gene existed. We analysed the expression of the CIDE-3 gene in human samples including adipose, breast tissues and some cell lines, namely hepatocellular carcinoma cell line HepG2, cervical cancer cell line HeLa, malignant melanoma cell line A375, tongue squamous cell carcinoma cell line Tca8113 and breast cancer cell line MCF-7, by RT-PCR. The results showed that the gene for CIDE-3 was expressed in all samples tested except for the MCF-7 and HeLa cell lines (Figure 5A) and that two transcripts existed. We cloned and sequenced these two transcripts from the human breast sample. The larger transcript was representative of the gene for CIDE-3; the smaller transcript lacked nt 194–357 of CIDE-3 and encoded CIDE-3 amino acids 75–238 (Figure 1A). This finding clearly demonstrated that alternative splicing of the gene

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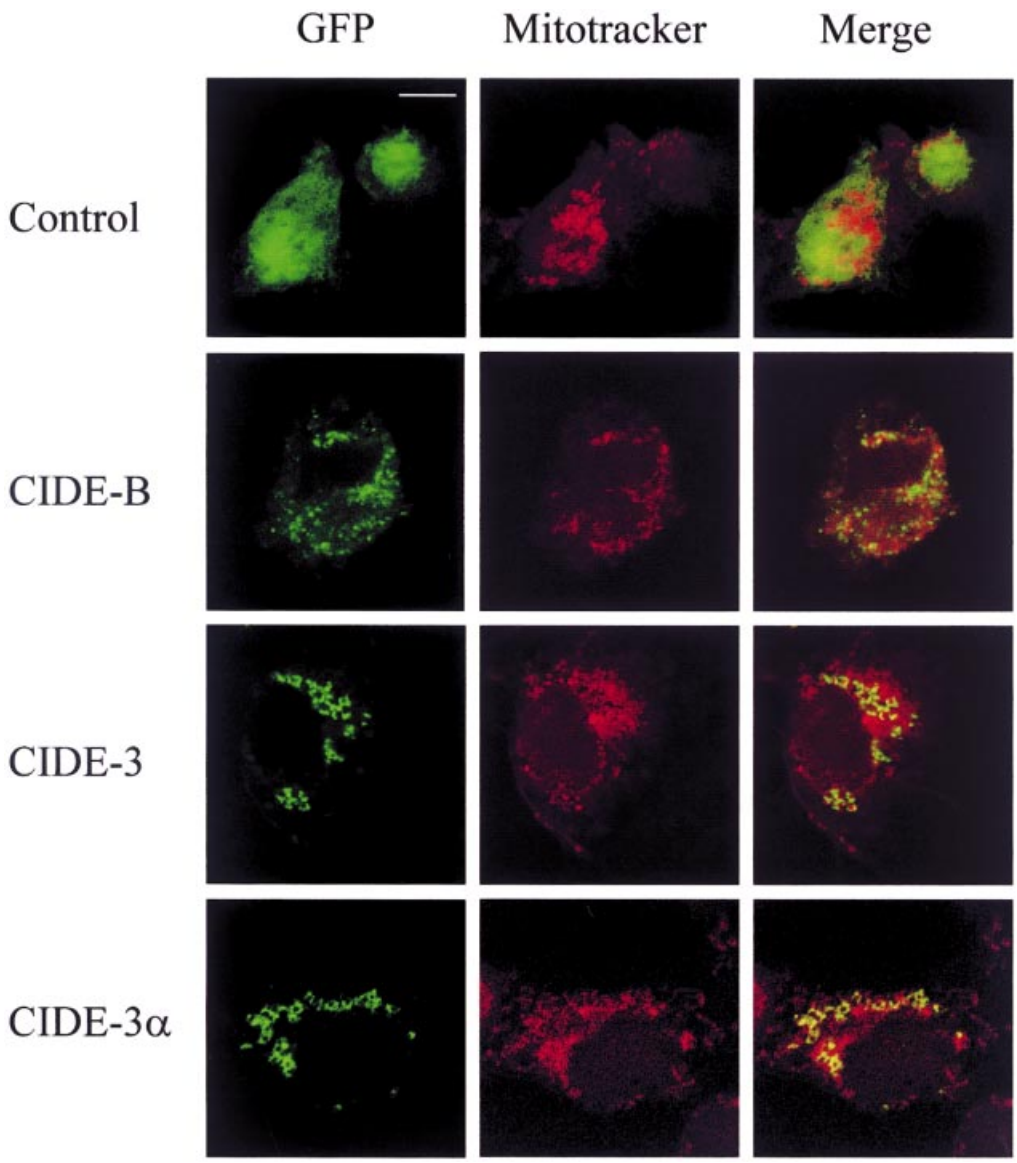

Figure 3 Subcellular localization of CIDE-B, CIDE-3 and CIDE-3α in COS-1 cells

COS-1 cells were transiently transfected with GFP linked to CIDE-B, CIDE-3 and CIDE-3α, respectively, stained with Mitotracker Red CMXRos 23 h after transfection and visualized by confocal microscopy at the appropriate wavelength for Mitotracker and GFP. The Mitotracker and GFP images were overlaid (merge). Scale bar, 7 μm.

for CIDE-3 existed, so we designated the smaller protein as CIDE-3α. After searching the GenBank database, we found that the sequence of CIDE-3α has been available in the database since the year 2000 (with accession number AK024530). But more information on this hypothetical protein was not reported until now.

We introduced an expression construct that encoded CIDE-3α into 293T cells. CIDE-3α could also induce both the condensation of nuclei (Figure 5B) and the fragmentation of DNA (Figure 5C), two characteristic features of apoptosis, as did CIDE-3. But the apoptosis-induced activity of CIDE-3α was weaker than that of CIDE-3 (Figure 5C). The expression of CIDE-3α in transfected cells was confirmed by Western-blot analysis (Figure 5D). We have also transiently transfected GFP-tagged CIDE-3α into COS-1 cells to determine its localization. The pattern presented by GFP-CIDE-3α was almost identical to that of GFP-CIDE-3 (Figure 3).

Structure and mapping of the gene for CIDE-3

By searching of human genomic sequence, it was discovered that the corresponding sequence of CIDE-3 was present in the genomic clone AC007783, which is approx. 169 kb long. This clone was sequenced and released to GenBank by Dr D. M. Muzny and co-workers (Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, U.S.A.). The analysis of genomic organization and alternative splicing showed that the CIDE-3 gene was composed of six exons that spanned approx. 13 kb (Figure 6A). The sequences of the splice junctions of the CIDE-3 gene were consistent with the GT-AG rule [19] (Figure 6B).

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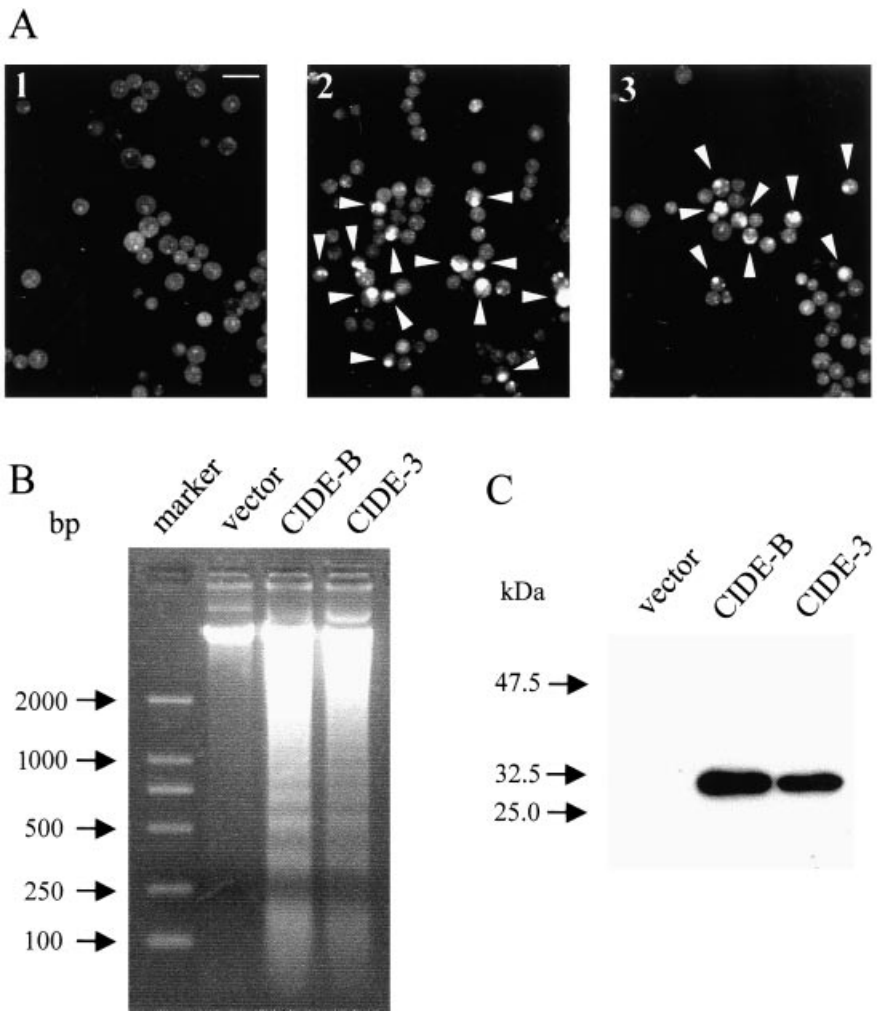

Figure 4 CIDE-3-induced apoptosis in 293T cells

(**A**) CIDE-3-induced condensation of nuclei in 293T cells. 293T cell were transfected with 1 μg of pcDNA3 (panel 1), pcDNA3-Flag-CIDE-B (panel 2) and pcDNA3-Flag-CIDE-3 (panel 3). Nuclei of transfected cells were stained with Acridine Orange and ethidium bromide at 18 h after transfection as described previously [16]. Scale bar, 40 μm. (**B**) DNA fragmentation assay. 293T cells were transfected with 5 μg of pcDNA3, pcDNA3-Flag-CIDE-B and pcDNA3-Flag-CIDE-3 as described in the Materials and methods section. The gel shows the fragmentation of DNA in response to CIDE-B and CIDE-3. (**C**) Analysis of the expression of CIDE-B and CIDE-3 proteins, by Western blotting, in cells transfected as in (**B**).

Table 1 Analysis of CIDE-3-induced apoptosis in CHO cells, demonstrating the dose-dependent effect, which was similar to that of CIDE-B

CHO cells (3×10^5) were co-transfected with the indicated amounts of plasmids for expression of FLAG-tagged CIDE-3 and FLAG-tagged CIDE-B together with a construct that encoded β-galactosidase. The total amount of DNA for each transfection was always 1.75 μg and was adjusted by addition of the appropriate amount of pcDNA3. The results (means $\pm$ S.D. for three experiments) represent percentages of apoptotic cells as a function of the total numbers of β-galactosidase-positive cells.

CHO-cell batch	Vector (μg)	CIDE-3 (μg)	CIDE-B (μg)	Apoptotic cells
1	1.5	–	–	20.8 $\pm$ 1.7
2	1.0	0.5	–	47.4 $\pm$ 2.3
3	0.5	1.0	–	60.6 $\pm$ 3.6
4	–	1.5	–	68.7 $\pm$ 5.8
5	1.0	–	0.5	56.3 $\pm$ 5.7
6	0.5	–	1.0	70.0 $\pm$ 6.1

From the results above (Figure 5A), we demonstrated that alternative splicing of CIDE-3 gene generated two transcripts, CIDE-3 and CIDE-3α. CIDE-3 comprised a full length ORF of the gene for CIDE-3. The transcript encoding CIDE-3α lacked exon 3 and its translation initiation site might be in exon 4. The alternative exon was confirmed by nucleotide sequencing.

AC007783 has been mapped to human chromosome 3p25 in the Map View issued at the NIH web site (http://www.ncbi.nlm.nih.gov). The region of chromosome 3p25 has been reported to be associated with loss of heterozygosity (LOH), which is deleted, at high frequency, in many tumour tissues. CIDE-3 located at this region may have some relations to tumorigenesis.

DISCUSSION

We have identified and characterized a novel human pro-apoptotic gene, CIDE-3, and its alternative splicing form

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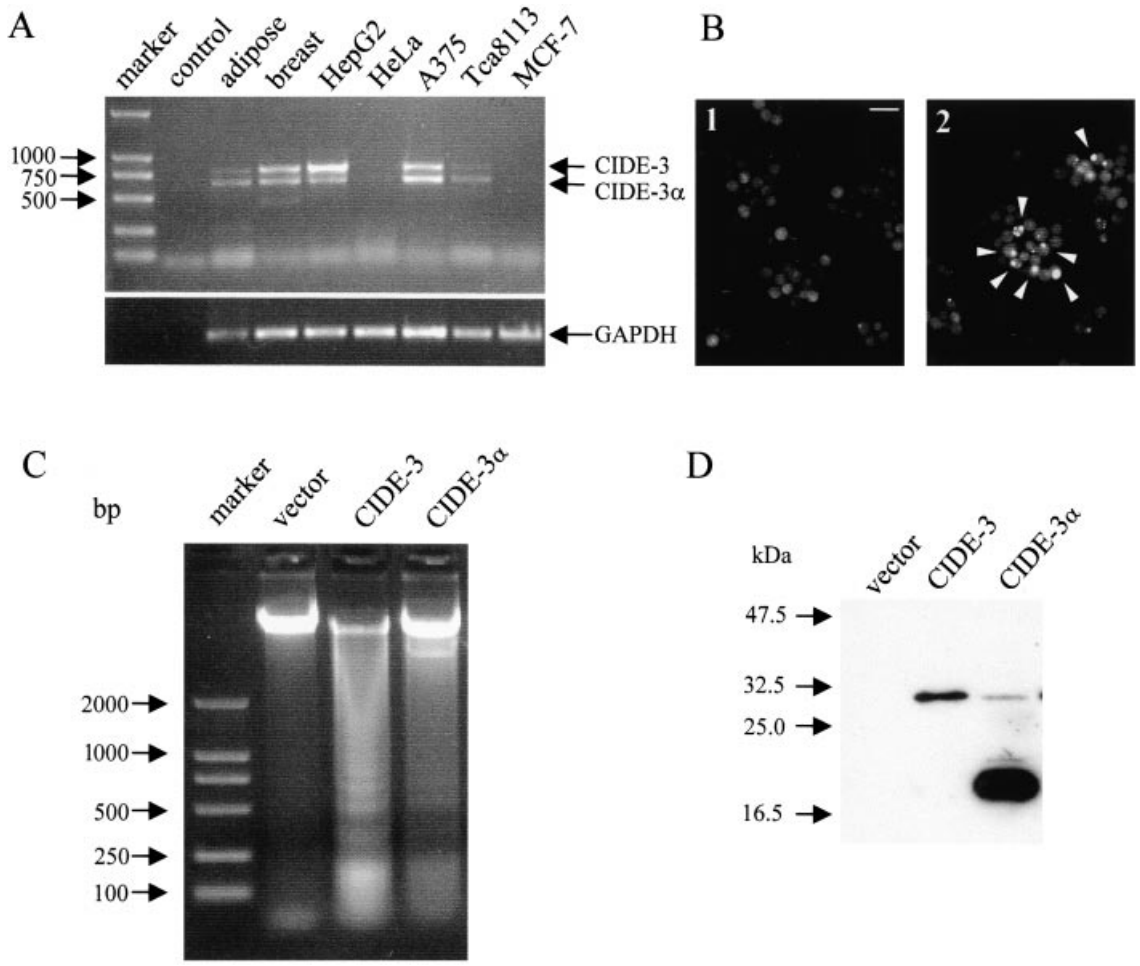

Figure 5 CIDE-3α-induced apoptosis in 293T cells

(**A**) Expression analysis, by RT-PCR, of CIDE-3 gene in human samples of adipose and breast tissues, and in human cell lines as indicated. All samples except MCF-7 and HeLa cell lines showed two transcripts indicated as CIDE-3 and CIDE-3α. (**B**) Ectopic expression of CIDE-3α-induced apoptosis of 293T cells. 293T cells were transfected with 1 μg of pcDNA3 (panel 1) and pcDNA3-Flag-CIDE-3α (panel 2). Nuclei of transfected cells were stained with Acridine Orange and ethidium bromide at 18 h after transfection. Scale bar, 40 μm. (**C**) DNA fragmentation assay. 293T cells were transfected with 5 μg of pcDNA3, pcDNA3-Flag-CIDE-3 and CIDE-3α as described in the Materials and methods section. The gel shows the fragmentation of DNA in response to CIDE-3 and CIDE-3α. (**D**) Western-blot analysis of the expression of CIDE-3α protein in the cells analysed in (**C**).

CIDE-3α. The CIDE-3 protein belongs to the CIDEs family and contains 238 amino acids. Northern-blot analysis showed that the CIDE-3 gene was expressed mainly in human small intestine, heart, colon and stomach, while CIDE-B was strongly expressed in liver and small intestine and at a lower level in colon, kidney and spleen. According to a previous report [11], CIDE-A was expressed mainly in the heart, and at a lower level in skeletal muscle, brain, lymph nodes, thymus, appendix and bone marrow. We concluded that the expression pattern of CIDE-3 was different from that of CIDE-A and CIDE-B.

The gene for CIDE-3 was determined to be a human homologue of mouse gene for FSP27. According to the report of Danesch et al. [18], the gene for FSP27 was an adipocyte-specific marker gene. In the present study, we clearly demonstrated that the gene for CIDE-3 was expressed not only in adipose tissues, but also in many other tissues and cell lines, such as hepatocellular carcinoma cell line HepG2 and malignant melanoma cell line A375. Our data indicated that the gene for CIDE-3 was not an adipocyte-specific gene.

The molecular mechanism by which CIDEs induce apoptosis is unclear. The apoptotic activity of CIDE-A is unaffected by broad-spectrum inhibitors of caspase [11]. CIDE-B has been reported to be located in mitochondria and formed high-affinity homo- and hetero-dimeric complexes, which are required for CIDE-B-induced apoptosis [14]. But according to our data (Figure 3), we are not able to observe a mitochondrial pattern for CIDE-B. It remains to be determined whether or not the CIDE-B induces apoptosis by directly acting on mitochondria. CIDE-3 was present in some cytosolic corpuscles that were not overlapping with mitochondria; it might trigger apoptosis by localization in other organelles. The molecular mechanism by which CIDE-3 induces apoptosis remains to be clarified.

Alterative splicing in this gene generated two isoforms, the longer mRNA transcripts (CIDE-3, six exons) and a shorter one (CIDE-3α, five exons). Skipping of exon 3 leads to production of CIDE-3α, which lacks 74 amino acids at the N-terminus. The alterative splicing did not influence subcellular localization of CIDE-3α. CIDE-3α showed the same pattern of subcellular

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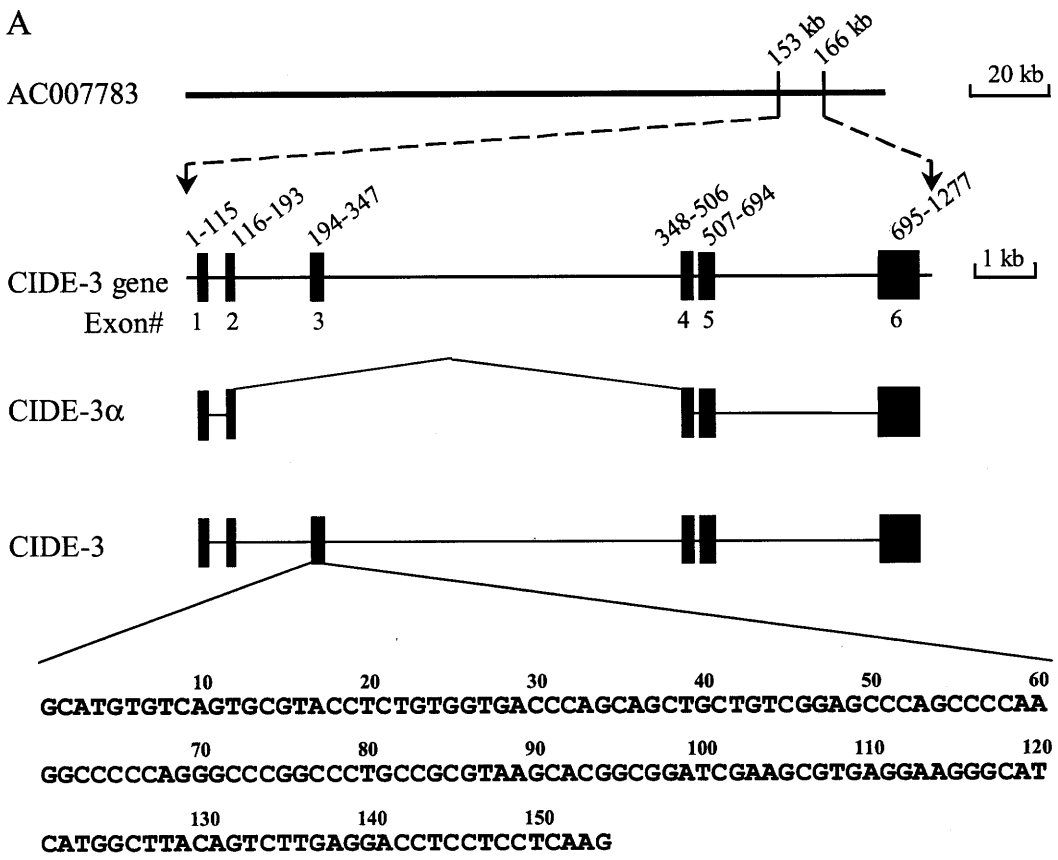

B

Exon		Sequence at exon/intron junction		
No.	Size (bp)	5' splice donor	3'splice acceptor	Intron size (bp)
1	>115	CTAGAG^{115}/gtgagggaac	tccttcccag/G^{116}TCCAA	430
2	78	CTCCAG^{193}/gtgaggaaag	actgccccag/G^{194}CATGT	1214
3	154	CTCAAG^{347}/gtgagagcac	tattccccag/G^{348}TCCGG	6735
4	158	GAACAG^{506}/gtgaggtccc	ctcaatgcag/G^{507}GGACA	94
5	188	CATGAA^{694}/gtgagcaaat	ttccacccag/G^{695}GAAGC	2585
6	>584	AAAAAA^{1305}		

Figure 6 The structure and alternative splicing of the gene for CIDE-3

Genomic organization and alternative splicing of the gene for CIDE-3. The first and last nucleotides in each exon are indicated by a pair of numbers that refer to their positions in the cDNA. The sequence of AC007783 is depicted as a solid line. The alternative exon was confirmed by nucleotide sequencing. (**B**) Exon-intron boundaries in the gene for CIDE-3. Exon sequences are in uppercase and intron sequences are in lowercase.

localization as CIDE-3. The apoptosis-induced activity of CIDE-3α was weaker than that of CIDE-3. Thus, these observations suggested that alterative splicing in the CIDE-3 gene, generating different protein isoforms, might be a mechanism for regulation of CIDE-3 activity. Notably this mechanism favours the theory that the synthesis of CIDE-3 isoforms could regulate their capacity to interact with unknown proteins which are involved in cell apoptosis.

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The chromosome 3p25 region of the human genome was associated with LOH and was deleted at a high frequency in oral squamous cell carcinoma [20], breast carcinoma [21,22], renal cell carcinoma [23–25], bladder tumour [26], pancreatic acinar cell carcinoma [27] and cervical cancer [28–30]. The location of the gene for CIDE-3 at chromosome 3p25 implicated that CIDE-3 might play an important role in prevention of tumorigenesis.

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