Identification and Characterization of DEDD2, a Death Effector Domain-containing Protein*

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A novel Death Effector Domain-containing protein was identified, DEDD2, which is closest in amino acid sequence homology to death effector domain-containing DNA-binding protein, DEDD. DEDD2 mRNA is expressed widely in adult human tissues with highest levels in liver, kidney, and peripheral blood leukocytes. DEDD2 interacts with FLIP, but not with Fas-associated death domain (FADD) or caspase-8. Overexpression of DEDD2 induces moderate apoptosis and results in substantial sensitization to apoptosis induced by Fas (CD95/APO-1), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, Apo2L), or FADD. In contrast, Bax- or staurosporine-mediated cell death is not affected by expression of DEDD2. Fluorescence microscopy showed that overexpressed DEDD2 translocates to the nucleus, which is dependent on the presence of a bipartite nuclear localization signal in the DEDD2 protein. Mutagenesis studies revealed that the translocation of the DED of DEDD2 to the nucleus is essential for its pro-apoptotic activity. These findings suggest that DEDD2 is involved in the regulation of nuclear events mediated by the extrinsic apoptosis pathway.

The initiation of death receptor-mediated apoptosis is characterized by the formation of signaling complexes that involve proteins containing the Death Domain (DD) and Death Effector Domain (DED). Upon activation of death receptors such as Fas, the cytoplasmic adapter molecule FADD interacts with the intracellular regions of the receptor proteins by homotypic binding of their DDs (1). Transduction of the apoptotic signal then depends on interaction of the DED in FADD with corresponding DEDs in pro-caspase-8, or pro-caspase-10, resulting in a protein complex that promotes trans-proteolysis of receptor-associated caspase pro-enzymes (2). The association of Fas (CD95/APO-1), FADD, and pro-caspase-8 has been termed “death-inducing signaling complex” (DISC) (3). The active caspase subunits are then released into the cytosol, where they cleave various substrates, including other caspases (e.g. caspase-3 (4)), cytoskeletal proteins (e.g. plectin (5)), or apoptosis effectors such as Bid (6, 7). Finally, the late stages of apoptosis are characterized by DNA fragmentation, which can be accomplished by endonucleases, such as caspase-activated DNase (8), and nuclear fragmentation associated with cleavage of nuclear scaffold proteins such as lamin (9).

Besides the crucial role of DED proteins in the formation of the DISC and the activation of DED-containing initiator caspases, DEDs in viral or cellular proteins also have the ability to inhibit apoptosis. The proteins v-FLIP and c-FLIP (FLICE-inhibitory proteins) bind to the DEDs of pro-caspase-8 and -10, interfering with their activation in the DISC (10). This DED-mediated anti-apoptotic mechanism may be of particular importance, because overexpression of FLIP has been reported in several types of cancer (11, 12).

However, DED-containing proteins affect apoptotic signaling in addition to interfering with initial events of apoptosis, such as the DISC formation. In this regard, Stegh et al. (13) identified a protein named DEDD (death effector domain-containing DNA-binding protein), which contains a DED and two nuclear localization signals and binds strongly to DNA. DEDD was shown to translocate from the cytosol to the nucleus after induction of apoptosis, thereby representing a possible novel mechanism for linking cytoplasmic and nuclear events during apoptosis. We now report the identification of a second nucleus-targeting, DED-containing protein, which we call DEDD2. The DEDD2 protein is a close relative of DEDD, sharing 48.5% amino acid sequence identity. DEDD2 resembles DEDD in its subcellular localization and has comparable pro-apoptotic activity but seems to differ from DEDD in its binding to other DED family members.

MATERIALS AND METHODS

Cloning of DEDD2 cDNA—TBLASTN searches of the human expressed sequence tag (EST) data base using the amino acid sequence of DEDD as a query resulted in the identification of two EST clones with homologous partial cDNAs. One of the clones (GenBank™ accession number AA218681) was kindly provided by the Genome Sequencing Center (St. Louis, MO) and sequenced in its entirety, revealing an open reading frame (ORF) encoding a 326-amino-acid protein with homology to DEDD, which we termed "DEDD2." The DEDD2 sequence was submitted to GenBank™ with the accession number AF443591. The corresponding genomic sequence for this cDNA was identified in the human genome data base (clone CIT-B-147B23), which was derived from chromosome 19q13.2.

Northern Blotting and RT-PCR Analysis—Northern blot membranes containing poly(A)+ RNA from multiple tissues (MTN, CLONTECH) were hybridized with a 32P-labeled DNA probe spanning the first 518 bp of the DEDD2 cDNA. Hybridization was performed according to the manufacturer’s instructions. For RT-PCR, we employed multiple tissue cDNA panels (CLONTECH) containing first-strand cDNA generated from eight different tissues. PCR was performed with the following primers: 5' primer, 5'-GGTGCCTCCAGAAGCT-3'; 3' primer, 5'-AGA-
AGGGCTTCAGA-3', and β-actin primers. Additionally, total RNA was isolated from different cell lines using the RNeasy Mini kit (Qiagen) according to a standard protocol.

**Plasmid Construction**—The ORF encoding DEDD2 was PCR-amplified from cDNA of the EST clone AA218861 using the forward primer, 5'-CGGCTGGAGCTCTGTCGGGCGGACTAC-3' and reverse primer, 5'-CGGCTGGAGCTCTGTCGGGCTGACC-3'. The PCR products were digested with EcoRI and XhoI and cloned into the EcoRI and XhoI sites of pcDNA3 (Stratagene). Epitope-tagged expression plasmids were constructed by PCR using primers flanking with FLAG or HA epitope tag sequences and appropriate restriction sites. GFP-DED2 and DEDD2-GFP was constructed by subcloning DEDD2 into the EcoRI and SalI sites of pcDNA3-myc-FLIP and pcDNA3-HA-FADD, respectively. The cDNAs encoding various fragments of DEDD2 were generated by PCR from the plasmid pcDNA3-DED2 using the following forward (F) and reverse (R) primers containing EcoRI and XhoI restriction sites and the sequence encoding the FLAG epitope tag: N1-DEDD2, 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (F) and 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (R); N3-DEDD2, 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (F) and 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (R); C1-DEDD2, 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (F) and 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (R); C2-DEDD2, 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (F) and 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (R); C3-DEDD2, 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (F) and 5'-CGGATCCATTGATCTAAGAAAGATGACGGACGGCGCTATCCGGGTCGACC-3' (R). To generate the nuclear-targeted DEDD2 proteins, DEDD2 fragments were cloned into the EcoRI and XhoI sites of pcDNA3-NLS (generated by the insertion of an oligonucleotide encoding for SV40-Large-T-like nuclear localization signal into EcoRI and XhoI sites of pcDNA3) (14).

**Cell Culture, Transfections, and Apoptosis Assays**—HEK293, COS-7, and LN-18 cells were maintained in Dulbecco's medium (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics. For transient transfection apoptosis assays, cells in six-well plates were transfected with pcDNA3 or pcDNA3-neo, and at 24 h post-transfection, cells were harvested. For apoptosis assays, 5 × 10⁴ cells per well were plated in 96-well plates, cultured for 24 h, and then incubated with anti-Fas CH11 antibody (MBL), TRAIL (BioTeK Instruments), or staurosporine (Sigma Chemical Co.) for 24 h in serum-free medium. The percentage of surviving cells was determined by 10 min in crystal violet solution (2%) in 20% methanol. The plates were washed three times with PBS, incubated with Alexa 594 goat anti-mouse IgG (CLONTECH), and then washed three times with PBS, incubated with Alexa 594 goat anti-mouse IgG (CLONTECH), and then washed three times with PBS. Subsequently, coverslips were mounted onto the slides using Mowiol (Hoechst). The cells were imaged by confocal microscopy using a Bio-Rad MRC 1024 instrument.

**RESULTS**

**Identification and Sequence Analysis of DEDD2**—Using the sequences of several Death Effector Domains (DEDs), the publicly available EST data bases were searched by saturated BLAST for the existence of new DED-containing proteins (17). This search identified two human EST clones (AW449244 and AA218861) that contained a DED with greatest sequence similarity to the DED of DEDD (15). One of the clones spanned a 2.0-kb sequence that encoded an open reading frame (ORF) corresponding to a 326-amino-acid protein, which we termed DED2. The CDNA sequence of DEDD was confirmed by amplifying DEDD2 cDNAs from the neuronal precursor cell line NT2. The predicted ORF was initiated by an AUG start codon within a favorable Kozak context. In addition to the DED, the predicted DEDD2 protein contains two nuclear localization signals (NLS) (Fig. 1A). The first one is identical to the NLS of another protein, AmidA (18), and the second is a typical bipartite NLS. Overall, DEDD2 shares 48.5% amino acid sequence identity and 55.3% DNA sequence identity with human DEDD (Fig. 1B and C). The DEDD2 gene is located on chromosome 19 (map 19q13.2), as determined by in silico screening of the human genome data base at NCBI. In contrast, the DEDD gene is located on chromosome 1q.

**DEDD2 mRNA Is Widely Expressed in Adult Human Tissues and Cell Lines**—Northern blotting demonstrated the presence of DEDD2 transcripts of ~2.0-kb length in most adult human...
tissues (Fig. 2A). High levels of DEDD2 mRNA were found in liver, kidney, heart, skeletal muscle, and peripheral blood leukocytes, whereas DEDD2 mRNA was absent or low in colon and small intestine. A 4.4-kb transcript was found in some tissues, and an additional, smaller 1.6-kb transcript was detected in RNA from peripheral blood leukocytes. RT-PCR assays using primers specific for DEDD2 extended and confirmed the Northern blot findings. High expression was found in peripheral blood leukocytes and ovary, whereas no expression was found in colon and small intestine (Fig. 2B, upper panel).

All of the human cell lines examined were positive for DEDD2 mRNA expression (Fig. 2B, lower panel).

**DEDD2 Binds to FLIP but Not to FADD or Caspase-8**—The DEDD2 protein was tested for interaction with other DED-containing proteins by in vitro binding assays and co-immunoprecipitations. First, binding experiments were performed by incubating a GST fusion protein containing the DED of DEDD2 or a GST-control fusion protein (GST-CD40) with various 35S-labeled, in vitro-translated DED-containing proteins (Fig. 3A). Although GST-DEDD2(DED) bound to the isolated DEDs of FADD and pro-caspase-8, no interaction was detectable with the full-length proteins. In contrast, GST-DEDD2(DED) readily bound to in vitro translated full-length FLIP. DEDD2 did not associate with itself, nor with the DED (like)-containing proteins DEDD, DAP3, Bap31, PEA-15, or BAR.

To confirm these findings in a more physiological setting, we performed co-immunoprecipitation experiments, wherein DEDD2, FADD, caspase-8, and FLIP were expressed in HEK293T cells with various epitope tags. In accordance with the in vitro binding assays, these studies indicated that DEDD2 binds to FLIP but not to FADD or pro-caspase-8 (Fig. 3B, left panel). These findings were also confirmed by using either N- or C-terminal FLAG-tagged DEDD2 proteins (Fig. 3B and not shown). As a comparison, we performed similar experiments with DEDD (Fig. 3B, right panel). Interestingly, DEDD was capable of binding FADD and, to a lesser degree, FLIP, but not caspase-8.

**DEDD2 Is a Weak Inducer of Apoptosis and Sensitizes Cells to Death Receptor- and FADD-mediated Cell Death**—To assess the effects of DEDD2 on apoptosis, HEK293N cells were transiently transfected with plasmids encoding wild-type DEDD2, GFP-DEDD2, DEDD2-GFP, FLAG-DEDD2, DEDD2-FLAG, and HA-DEDD2. Ectopic expression of DEDD2 resulted in weak induction of apoptosis after 24 h, whereas at 72-h post-transfection the extent of apoptotic cell death reached a maximum with ~20% dead cells (Fig. 4A). The percentage of cell death was similar with all constructs irrespective of the epitope tag. As a control, the cells were transfected with a plasmid encoding DEDD-FLAG, resulting in a comparable extent of apoptosis (15–20%; data not shown).

Given the presence of a DED, we wondered if DEDD2 would interfere with DD/DED-related apoptosis signaling. Thus, we co-transfected HEK293N cells with FADD and DEDD2, or the empty control vector (neo). In fact, DEDD2 transfection substantially sensitized the cells to FADD-induced cell death (Fig. 4B), whereas DEDD2 did not enhance Bax-mediated apoptosis.
To further examine DEDD2-mediated effects on apoptosis, we stably transfected the Fas- and TRAIL-sensitive human glioblastoma cell line LN-18 with a plasmid encoding DEDD2-FLAG. Treatment of these cells with anti-Fas antibody CH11 or TRAIL demonstrated increased sensitivity for death receptor-mediated apoptosis of DEDD2 transfectants as compared with control cells (Fig. 4, D, E, G, H). CH11- and TRAIL-induced effects are depicted either as the fraction of surviving cells (Fig. 4, D and E) or as the percentage of apoptotic cells as determined by DAPI staining (Fig. 4, G and H). In contrast, transfectants and control cells were equally susceptible to staurosporine-induced cell death (Fig. 4, F and I).

Given the prolonged time course of DEDD2-induced cell death (Fig. 4A), we sought to confirm that DEDD2-mediated cell death is truly apoptotic cell death. COS-7 cells were transiently transfected with different DEDD2-encoding plasmids, fixed at various times after transfection, stained, and subjected to fluorescence microscopy. Fig. 5 shows the typical staining...

Fig. 2. Expression of DEDD2 in human tissues and cell lines. A, DEDD2 mRNA expression in human tissues. A Northern blot of RNA from multiple tissues was probed with a radiolabeled human DEDD2 cDNA probe encoding the first 518 bp. The same membrane was reprobed with a radiolabeled β-actin probe (a, skeletal muscle; b, small intestine; c, peripheral blood leukocytes). B, first-strand cDNA from various adult human tissues and from human cancer cell lines was PCR amplified using primers specific for DEDD2 (d, DEDD2 plasmid cDNA; e, hepatocellular carcinoma cell line Alexander; f, DEDD plasmid cDNA).

Fig. 3. Interaction of DEDD2 with other DED-containing proteins. A, in vitro protein binding assays were performed using GST-DEDD2 (containing the DED) or control GST-CD40 (cytosolic domain) fusion proteins. The fusion proteins were immobilized on glutathione-Sepharose and then incubated with in vitro translated 35S-labeled proteins as indicated. B, HEK293T cells were transiently transfected with various combinations of plasmids encoding DEDD2-FLAG, DEDD-FLAG, HA-FADD, myc-FLIP, and HA-pro-caspase-8. Cell lysates were prepared and immunoprecipitations (IP) were performed using either anti-myc or anti-HA monoclonal antibodies, followed by SDS-PAGE/immunoblot analysis (blot) using antibodies specific for the FLAG or myc tag. As a control (first four lanes of each panel), an aliquot of the lysates was also loaded directly into gels and analyzed by immunoblotting. The interaction of FLIP and pro-caspase-8 served as a positive control (bottom panels).
pattern of cells transiently transfected with plasmids encoding FLAG-, HA-, or GFP-tagged DEDD2. DEDD2 localizes to the nuclei of cells with accumulation in nucleoli-like structures, whereas virtually no staining is detectable in the cytoplasm (Fig. 5, A and B). HEK293N cells were co-transfected with plasmids encoding GFP (used as a marker for transfection efficiency), DEDD2, or neo control vector, and plasmids encoding FADD (B) or Bax (C) at concentrations as indicated. At 24 h post-transfection, cells were collected and stained with DAPI. The percentage of GFP-positive cells with apoptotic morphology (fragmented nuclei or condensed chromatin) was determined. D–I, the LN-18 human glioma cell line stably expressing DEDD2-FLAG was generated as outlined under “Materials and Methods.” DEDD2-expressing cells and control cells transfected with the empty control vector (neo) were treated with various concentrations of anti-Fas antibody CH11 (D, G), TRAIL (E, H), or staurosporine (F, I), as indicated. After 24 h, the relative number of surviving cells was assessed by crystal violet assay, and the data were expressed as a percentage relative to control (untreated) cells (D–F). Alternatively, apoptotic cell death of LN-18 glioma cells was directly determined by staining the cells with DAPI, counting cells exhibiting nuclear condensation and/or fragmentation, and expressing the data as a percentage (mean ± S.D.; n = 3) (G–I). As an expression control, lysates of stable DEDD2 transfectants and control cells were subjected to an immunoblot analysis using an anti-FLAG antibody (see inset in D).

Targeting of the Death Effector Domain to the Nucleus Is Required for the Pro-apoptotic Activity of DEDD2—To further examine which regions of DEDD2 are responsible for induction of cell death, we generated a series of N- and C-terminal truncation mutants of DEDD2, performed transient transfections in HEK293N cells, and screened for cell death (Fig. 6A). Importantly, only mutants harboring the DED and the bipartite NLS retained pro-apoptotic activity. In contrast, protein fragments containing the DED only, the DED with the first NLS, or the complete C terminus (irrespective of the presence of a NLS) were non-toxic (Fig. 6A, right column). Immunoblot analysis confirmed that all DEDD2 protein fragments were produced at comparable levels, thus excluding trivial explanations for the observed differences in function (Fig. 6B).

Next, we asked how the differences in apoptotic activity of the mutants corresponded to their subcellular localization. To this end, COS-7 cells were transfected with the mutants, fixed, stained, and examined by fluorescence microscopy. Full-length DEDD2 and N3-DEDD2, which lacks the C terminus, clearly localized into the nucleus (Fig. 7, A and B). C1-DEDD2, which consists of the C terminus region only, was found exclusively in the cytoplasm (Fig. 7C), whereas C2-DEDD2 that contains the C terminus region of DEDD2 and the bipartite NLS localized...
Apolipoprotein E (ApoE) Deficiency in the Liver—Part 2: Effects on Lipid Metabolism

Introduction

ApoE deficiency has been shown to have significant effects on lipid metabolism in the liver, including changes in lipid synthesis and secretion. In patients with ApoE deficiency, the levels of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) are reduced, while high density lipoprotein (HDL) levels are increased.

Materials and Methods

In this study, ApoE-deficient mice were fed a high-fat diet for 12 weeks to induce liver disease. Liver tissue was collected and analyzed for lipid content, gene expression, and protein levels using qPCR and Western blotting.

Results

Liver lipid analysis revealed a significant increase in triglyceride levels in ApoE-deficient mice compared to wild-type controls. Gene expression analysis showed upregulation of genes involved in lipid synthesis and downregulation of genes involved in lipid metabolism. Western blots confirmed the increased expression of fatty acid synthase (FAS) and acyl-CoA synthetase (ACS) in ApoE-deficient mice.

Discussion

The results of this study demonstrate the significant effects of ApoE deficiency on lipid metabolism in the liver. These findings highlight the importance of ApoE in lipid homeostasis and suggest potential targets for future therapeutic interventions.

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Fig. 5. Subcellular distribution of DEDD2. A and B, COS-7 cells were transfected with pEGFP-C2-DEDD2 and subjected to immunofluorescence after 16 h. GFP-DEDD2 typically localizes to the nucleus and accumulates in nucleoli-like structures (A). The size and form of the cytoplasm is shown in the Nomarski phase-contrast image (B). C, the apoptotic nature of DEDD2-induced cell death was confirmed by immunofluorescence. COS-7 cells were transfected with a plasmid encoding DEDD2-FLAG and stained with an anti-FLAG antibody after 24 h. The panel shows the nuclear morphology of a representative transfected cell undergoing apoptosis. D-F, co-localization of DEDD2 with nucleophosmin. COS-7 cells were transfected with pEGFP-C2-DEDD2 (D) and stained for nucleophosmin using a monoclonal anti-nucleophosmin antibody followed by an Alexa 594-labeled secondary antibody (E). The overlay of both images is shown in the bottom panel (F).

DISCUSSION

Multiple DED-containing proteins that modulate apoptosis have been identified (20). DEDs are found in the pro-forms of the initiator caspases, caspase-8 and -10, in the adaptor protein FADD, and in FLIPs, among other proteins. Viral and cellular FLIPs inhibit Fas-induced apoptosis by interaction with the two tandem DEDs of pro-caspase-8 and by competing with pro-caspase-8 and -10 for binding to FADD. Other proteins that contain DEDs or DED-like domains are PEA-15 (21, 22), Flash (23), Bap31 (24), BAR (25), and HIP-1 (26). Interestingly, it has also been suggested that DEDs are involved in cellular signaling events other than the regulation of apoptosis, such as the activation of the extracellular signal receptor-activated kinase by the DED of PEA-15 (27). Thus, apart of their involvement in the initial events of death receptor-dependent apoptosis at the DISC, DED-containing proteins may also play other important roles in cellular signaling.

In this regard, the identification of DEDD contributed substantially to an extended view of the possible functions of DEDs. DEDD, which contains an N-terminal DED and a C-terminal histone-like domain, is present in the cytosol but translocates to nuclei during Fas-induced apoptosis (13). There, it supposedly shuts off ribosomal RNA gene transcription by repressing both RNA polymerase I and II transcription. DEDD is therefore an apoptosis-regulating protein that possibly links early and late events involved in apoptosis. Interestingly, it has also been suggested that DEDD mRNA is up-regulated after induction of apoptosis in germ cells (28).

We report here the identification of a new protein, DEDD2, that contains a DED and is capable of inducing apoptosis upon translocation to the nucleus. The amino acid sequence and domain structure of DEDD2 closely resembles DEDD. Like DEDD, the DEDD2 gene is widely expressed in human tissues and cell lines and is highly conserved between human and mouse (data not shown). When expressed transiently, both DEDD and DEDD2 induce apoptosis to a similar extent. Cell death induced by overexpressed DEDD2 occurs with a protracted time course, with the percentage of apoptotic cells reaching a maximum after 3 days. Moreover, the translocation of the DED of DEDD2 to the nucleus, most probably accomplished by the bipartite NLS, is crucial for induction of apoptosis. These findings suggest a role for DEDD2 in late apoptotic events that affect the cell nucleus. In this regard, the apoptotic cell death program is known to affect the nuclear matrix (or scaffold), which is mainly composed of non-histone proteins and RNA. Nuclear matrix proteins are cleaved during apoptosis thereby facilitating the breakdown of the nuclear scaffold and, finally, of the entire nucleus. These include topoisomerase IIa, NuMA, SATB1, and lamins (29). Interestingly, preliminary findings obtained by cDNA microarray analysis indicate that the expression of various scaffold proteins is decreased in cells overexpressing DEDD2. Nuclear apoptotic events also affect DNA transcription and RNA processing, the latter of which is mainly localized in the nucleolus. For example, a central regulator of rDNA transcription, upstream binding factor, is cleaved during apoptosis (30). The striking accumulation of DEDD2 in nucleoli points to a possible interference with RNA transcription and processing, e.g., by promoting the cleavage of upstream binding factor or other nucleolar proteins. Apart from structural changes in the nucleoli, this would eventually lead to ribosomal dysfunction. Interestingly, death re-
ceptor-dependent degradation of 28 S ribosomal RNA has been shown to result in inactivation of ribosomes, which has a devastating effect on cellular homeostasis (31). Because the data presented here are derived from overexpression experiments, it will be crucial to study the nuclear effects of endogenous DEDD2 in more detail.

Despite the similarities between DEDD and DEDD2, which indicate that these two proteins are closely related, differences also exist that suggest non-redundant mechanisms of action. In contrast to DEDD, the DEDD2 protein failed to bind FADD, and DEDD2 showed stronger binding to FLIP than DEDD. The sequence differences within the DEDs of the two proteins may explain these distinct binding properties. Furthermore, the N-terminal mutants of DEDD2 have similar pro-apoptotic activity as the full-length protein, thus, the C terminus of DEDD2 may lack the autoinhibitory function reported for the corresponding region of DEDD. Other differences become evident when the functions of the NLSs are examined. Although the NLS1 of DEDD is reported to be responsible for nucleolar targeting, the NLS1 of DEDD2 was sufficient for nuclear targeting but did not result in nucleolar accumulation. In contrast, although the NLS2 of DEDD was shown to promote nuclear but

FIG. 6. DEDD2 truncation mutants and their apoptotic activity. A, the diagram of DEDD2 truncation mutants shows the position of the FLAG tag and the amino acid position of each domain within the DEDD2 sequence. The regions encoding the Death Effector Domain (black box) and the nuclear localization signal (gray box) are indicated. The results of transient transfection apoptosis assays are summarized, indicating which mutants did (+) or did not (−) induce at least a 20% increase of apoptosis in HEK293N cells. B, immunoblot analysis of DEDD2 mutants. The indicated plasmids were transiently transfected into HEK293N cells, and lysates were prepared, normalized for total protein content (20 μg), and subjected to SDS-PAGE immunoblot analysis with anti-FLAG antibody.
not nucleolar targeting, the bipartite NLS2 of DEDD2 resulted in strong nucleolar accumulation. These differences may be due to the sequence divergence of the two proteins. For example, in DEDD2, the NLS2 regions of DEDD and DEDD2 are only 40.9% identical in amino acid sequence.

In this report, we show that DEDD2 (as well as DEDD) has the ability to bind c-FLIP. It has been demonstrated that DEDD is not involved in the formation of the DISC (13). Therefore, one could speculate that DEDD and DEDD2 antagonize FLIP-mediated anti-apoptosis by sequestering FLIP to the nucleus, thus sensitizing the cell to death receptor-induced apoptosis. However, subcellular fractionation experiments revealed no substantial DEDD2-mediated relocation of FLIP to the nucleus (data not shown). DEDAF, a DED-binding protein, has recently been described (32). DEDAF binding to DEDD was observed in the nucleus, resulting in a change in subnuclear distribution of DEDD. It therefore will be interesting to explore the interaction of DEDD2 with this protein. Further investigation of this intriguing cell death pathway will shed light on the mechanisms responsible for linking DEDs and nuclear apoptotic events.

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Fig. 7. Fluorescence microscopy of DEDD2 and DEDD2 mutants. A–H, COS-7 cells were transfected with plasmids encoding full-length DEDD2 (A); N3-DED2, which lacks the C terminus but retains NLS1 and NLS2 (B); C1-DED2, which contains the C terminus only (C); C2-DED2, which contains the C terminus and the bipartite NLS2 (D); N1-DED2, which contains only the DED (E); NUC-N1-DED2, which contains the DED and the SV40-Large-T-like NLS (generated by subcloning the DED into pCDNA3-NLS) (F); N2-DED2, which contains the DED and NLS1 (G); and NUC-N2-DED2, which contains the DED, NLS1, and the SV40-Large-T-like NLS (H). At 16 h post-transfection, cells were fixed in 2% formaldehyde, stained with a monoclonal antibody recognizing the FLAG epitope, and visualized by laser confocal microscopy.
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