The EMBO Journal Vol.17 No.20 pp.5974–5986, 1998

DEDD, a novel death effector domain-containing protein, targeted to the nucleolus

Alexander H.Stegh, Olaf Schickling, Andreas Ehret, Carsten Scaffidi, Christoph Peterhänsel[1], Thomas G.Hofmann, Ingrid Grummt[1], Peter H.Krammer and Marcus E.Peter[2]

Tumor Immunology Program and [1]Division of Molecular Biology of the Cell II, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

[2]Corresponding author
e-mail: M.Peter@dkfz-heidelberg.de

The CD95 signaling pathway comprises proteins that contain one or two death effector domains (DED), such as FADD/Mort1 or caspase-8. Here we describe a novel 37 kDa protein, DEDD, that contains an N-terminal DED. DEDD is highly conserved between human and mouse (98.7% identity) and is ubiquitously expressed. Overexpression of DEDD in 293T cells induced weak apoptosis, mainly through its DED by which it interacts with FADD and caspase-8. Endogenous DEDD was found in the cytoplasm and translocated into the nucleus upon stimulation of CD95. Immunocytological studies revealed that overexpressed DEDD directly translocated into the nucleus, where it co-localizes in the nucleolus with UBF, a basal factor required for RNA polymerase I transcription. Consistent with its nuclear localization, DEDD contains two nuclear localization signals and the C-terminal part shares sequence homology with histones. Recombinant DEDD binds to both DNA and reconstituted mononucleosomes and inhibits transcription in a reconstituted *in vitro* system. The results suggest that DEDD is a final target of a chain of events by which the CD95-induced apoptotic signal is transferred into the nucleolus to shut off cellular biosynthetic activities.

Keywords: apoptosis/death effector domain (DED)/mononucleosome/nucleolus/transcriptional inhibition

Introduction

Recently, a new subfamily of the tumor necrosis factor (TNF) receptor superfamily, the death receptors, has been identified (Peter *et al.*, 1998). Death receptors such as TNF-R1, DR3 (APO-3/TRAMP/Wsl-1/LARD), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and CD95 (APO-1/Fas) are characterized by the presence of a death domain (DD) within the cytoplasmic region and have been shown to trigger apoptosis upon binding of their cognate ligands or specific agonistic antibodies. The first event which can be detected during apoptosis mediated by CD95 (APO-1/Fas) is recruitment of the first-level caspase-8 (FLICE/MACH/Mch5) to the death-inducing signaling complex (DISC) (Boldin *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Muzio *et al.*, 1996), this being observed within seconds of receptor crosslinking (Kischkel *et al.*, 1995). Binding of caspase-8 to the DISC is mediated by the adaptor molecule FADD/Mort-1 that contains a death effector domain (DED) by which it binds to caspase-8 (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995, 1996). Caspase-8 is then activated mainly by association with the DISC (Medema *et al.*, 1997), resulting in the release of active caspase-8 subunits which can then cleave various death substrates such as cytoskeletal proteins (e.g. plectin; A.H.Stegh *et al.*, manuscript submitted) or other caspases (e.g. caspase-3; Scaffidi *et al.*, 1998) that can in turn cleave a large number of intracellular death substrates. Other DED-containing proteins that have been suggested to be involved in signaling of death receptors include caspase-10 (Mch4/FLICE2) (Fernandes-Alnemri *et al.*, 1996; Vincenz and Dixit, 1997) and c-FLIP (FLAME/CASH/I-FLICE/Casper/Mrit/Clarp/Usurpin) (Goltsev *et al.*, 1997; Han *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Shu *et al.*, 1997; Srinivasula *et al.*, 1997; Rasper *et al.*, 1998).

The end point of apoptosis is DNA fragmentation in the nucleus. Between early morphological and nuclear changes a number of events can be detected depending on the type of apoptosis and the cell tested. Essentially all components of a cell are affected. Recently, an endonuclease, CAD, was cloned that was shown to be one of the DNA cleaving activities required for apoptosis (Sakahira *et al.*, 1998). CAD was found in complex with its inhibitor ICAD/DFF45 located in the cytoplasm (Liu *et al.*, 1997; Enari *et al.*, 1998). Upon apoptosis induction, ICAD is cleaved by a caspase and CAD was proposed to translocate to the nucleus where it exerts its activity as an endonuclease.

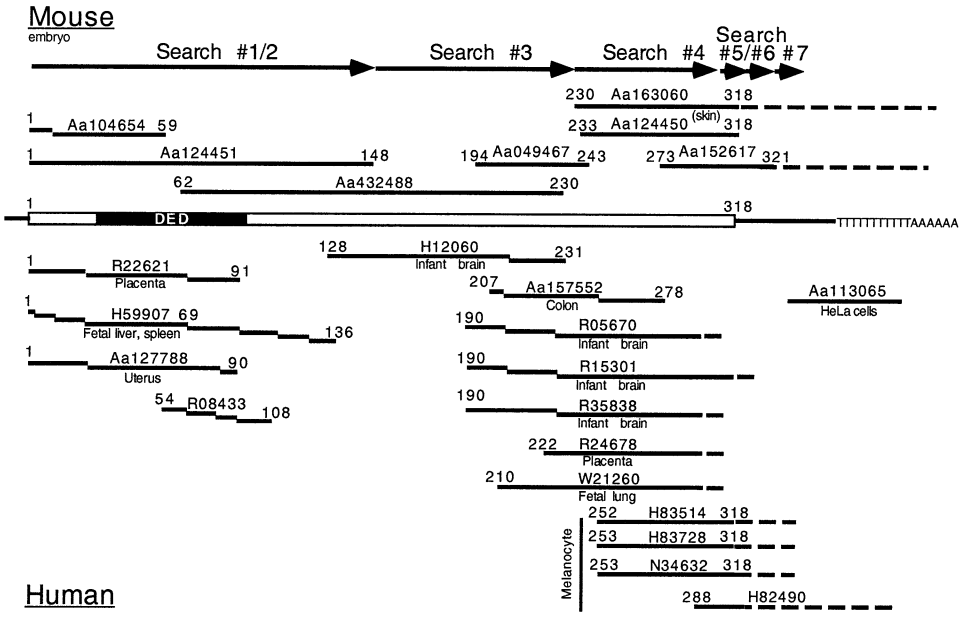
We now describe and functionally characterize a novel protein that contains an N-terminal DED with homology to the DED of FADD, c-FLIP, caspase-8 and caspase-10. This protein binds strongly to DNA with histone-like properties and was therefore called DEDD (for DED containing DNA-binding protein). DEDD weakly induced apoptosis and translocated to the nucleus during CD95-mediated apoptosis. This translocation was in part dependent on activation of caspases. Overexpressed DEDD localized exclusively to nucleoli and recombinant DEDD inhibited transcription of rDNA in a reconstituted *in vitro* assay. The results suggest that DEDD represents a new link between cytoplasmic and nuclear apoptotic events.

Results

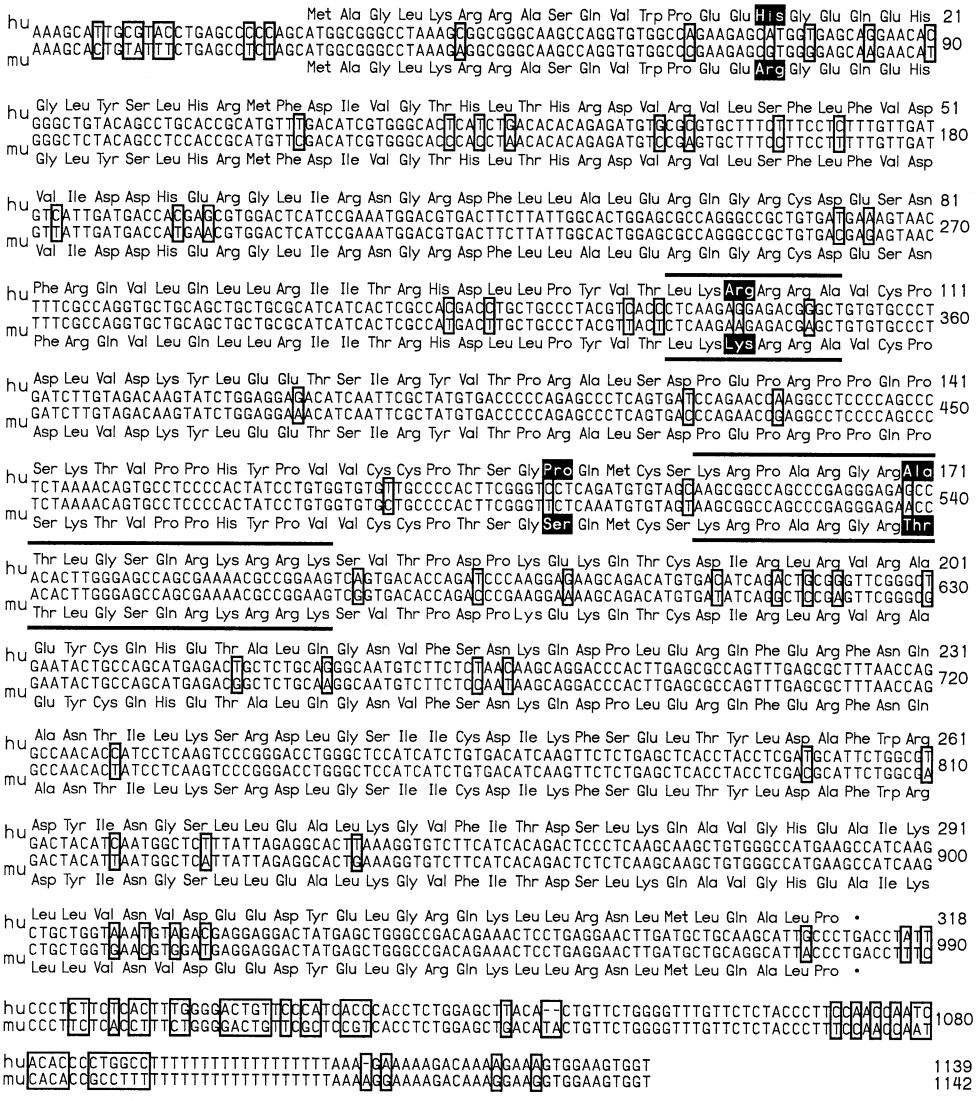
Cloning of full-length DEDD by 'EST walking'

Using the sequence of 22 different DEDs, an algorithm was generated to search the translated non-redundant union of EMBL and GenBank DNA databases for the

A


Mouse
embryo
Search #1/2
Search #3
Search #4
Search #5/#6 #7
230 Aa163060 318
1 Aa104654 59
233 Aa124450 (skin) 318
1 Aa124451 148
194 Aa049467 243
273 Aa152617 321
62 Aa432488 230
1
DED
318
TTTTTTTTTTAAAAAA
128 H12060 231
Infant brain
1 R22621 91
Placenta
207 Aa157552 278
Colon
Aa113065
HeLa cells
1 H59907 69
Fetal liver, spleen
136
190 R05670
Infant brain
1 Aa127788 90
Uterus
190 R15301
Infant brain
54 R08433 108
190 R35838
Infant brain
222 R24678
Placenta
210 W21260
Fetal lung
252 H83514 318
253 H83728 318
253 N34632 318
Melanocyte
288 H82490
Human

B


hu Met Ala Gly Leu Lys Arg Arg Ala Ser Gln Val Trp Pro Glu Glu His Gly Glu Gln Glu His 21
mu Met Ala Gly Leu Lys Arg Arg Ala Ser Gln Val Trp Pro Glu Glu Arg Gly Glu Gln Glu His
hu Gly Leu Tyr Ser Leu His Arg Met Phe Asp Ile Val Gly Thr His Leu Thr His Arg Asp Val Arg Val Leu Ser Phe Leu Phe Val Asp 51
mu Gly Leu Tyr Ser Leu His Arg Met Phe Asp Ile Val Gly Thr His Leu Thr His Arg Asp Val Arg Val Leu Ser Phe Leu Phe Val Asp
hu Val Ile Asp Asp His Glu Arg Gly Leu Ile Arg Asn Gly Arg Asp Phe Leu Leu Ala Leu Glu Arg Gln Gly Arg Cys Asp Glu Ser Asn 81
mu Val Ile Asp Asp His Glu Arg Gly Leu Ile Arg Asn Gly Arg Asp Phe Leu Leu Ala Leu Glu Arg Gln Gly Arg Cys Asp Glu Ser Asn
hu Phe Arg Gln Val Leu Gln Leu Leu Arg Ile Ile Thr Arg His Asp Leu Leu Pro Tyr Val Thr Leu Lys Arg Arg Arg Ala Val Cys Pro 111
mu Phe Arg Gln Val Leu Gln Leu Leu Arg Ile Ile Thr Arg His Asp Leu Leu Pro Tyr Val Thr Leu Lys Lys Arg Arg Ala Val Cys Pro
hu Asp Leu Val Asp Lys Tyr Leu Glu Glu Thr Ser Ile Arg Tyr Val Thr Pro Arg Ala Leu Ser Asp Pro Glu Pro Arg Pro Pro Gln Pro 141
mu Asp Leu Val Asp Lys Tyr Leu Glu Glu Thr Ser Ile Arg Tyr Val Thr Pro Arg Ala Leu Ser Asp Pro Glu Pro Arg Pro Pro Gln Pro
hu Ser Lys Thr Val Pro Pro His Tyr Pro Val Val Cys Cys Pro Thr Ser Gly Pro Gln Met Cys Ser Lys Arg Pro Ala Arg Gly Arg Ala 171
mu Ser Lys Thr Val Pro Pro His Tyr Pro Val Val Cys Cys Pro Thr Ser Gly Ser Gln Met Cys Ser Lys Arg Pro Ala Arg Gly Arg Thr
hu Thr Leu Gly Ser Gln Arg Lys Arg Arg Lys Ser Val Thr Pro Asp Pro Lys Glu Lys Gln Thr Cys Asp Ile Arg Leu Arg Val Arg Ala 201
mu Thr Leu Gly Ser Gln Arg Lys Arg Arg Lys Ser Val Thr Pro Asp Pro Lys Glu Lys Gln Thr Cys Asp Ile Arg Leu Arg Val Arg Ala
hu Glu Tyr Cys Gln His Glu Thr Ala Leu Gln Gly Asn Val Phe Ser Asn Lys Gln Asp Pro Leu Glu Arg Gln Phe Glu Arg Phe Asn Gln 231
mu Glu Tyr Cys Gln His Glu Thr Ala Leu Gln Gly Asn Val Phe Ser Asn Lys Gln Asp Pro Leu Glu Arg Gln Phe Glu Arg Phe Asn Gln
hu Ala Asn Thr Ile Leu Lys Ser Arg Asp Leu Gly Ser Ile Ile Cys Asp Ile Lys Phe Ser Glu Leu Thr Tyr Leu Asp Ala Phe Trp Arg 261
mu Ala Asn Thr Ile Leu Lys Ser Arg Asp Leu Gly Ser Ile Ile Cys Asp Ile Lys Phe Ser Glu Leu Thr Tyr Leu Asp Ala Phe Trp Arg
hu Asp Tyr Ile Asn Gly Ser Leu Leu Glu Ala Leu Lys Gly Val Phe Ile Thr Asp Ser Leu Lys Gln Ala Val Gly His Glu Ala Ile Lys 291
mu Asp Tyr Ile Asn Gly Ser Leu Leu Glu Ala Leu Lys Gly Val Phe Ile Thr Asp Ser Leu Lys Gln Ala Val Gly His Glu Ala Ile Lys
hu Leu Leu Val Asn Val Asp Glu Glu Asp Tyr Glu Leu Gly Arg Gln Lys Leu Leu Arg Asn Leu Met Leu Gln Ala Leu Pro • 318
mu Leu Leu Val Asn Val Asp Glu Glu Asp Tyr Glu Leu Gly Arg Gln Lys Leu Leu Arg Asn Leu Met Leu Gln Ala Leu Pro •
hu 1080
mu 1080
hu 1139
mu 1142

existence of new DED-containing proteins that might be involved in CD95-mediated apoptosis. This search identified a mouse EST clone (DDBJ\EMBL\GenBank accession number: Aa124451) that contained a DED homologous to the DED of FADD and caspase-8 (Peter *et al.*, 1997). Using the deduced protein sequence of this EST clone in TBlastN searches, other EST sequences containing overlapping identical nucleotide sequences were identified (Figure 1A). These EST sequences were used to search for more overlapping EST sequences. The correct reading frame was identified by comparing human and mouse EST sequences. Reading frames were aligned in a way that most exchanges between human and mouse EST sequences were in the wobble position. Seven such searches identified 23 EST sequences covering the full-length clones for both human and murine cDNAs. To confirm the existence and the identity of this open reading frame, the cDNA was amplified from a human (Jurkat) and a murine (EL4) cell line by reverse transcription–polymerase chain reaction (RT–PCR) and sequenced. Open reading frames in both man and mouse code for a 318 amino acid protein (Figure 1B). The protein was designated DEDD, for death effector domain containing DNA binding protein (see below). On the amino acid level, DEDD is 98.7% identical between man and mouse and three of the four amino acid exchanges represent replacements with homologous residues. On the DNA level, DEDD is 94.1% identical between man and mouse. On the RNA level, DEDD is expressed ubiquitously (Figure 2). All tissues tested express a 2.3 kb transcript (Figure 2A). In some tissues an additional transcript of 4.2 kb was found. Moreover, using RT–PCR, DEDD was found in all cell lines tested (Figure 2B), confirming that DEDD mRNA is expressed ubiquitously.

DEDD contains a DED and two nuclear translocation signals (NLS)

DEDD is a protein with mostly acidic domains (Figure 3A). Only two putative NLS (Figure 1B) and an area in between predominantly contain basic amino acids. Alignment of DEDD with proteins involved in death receptor signaling pathways revealed that DEDD contains a DED (amino acid positions 23–98) that is homologous to the DEDs of the human proteins FADD, caspase-8, caspase-10 and c-FLIP (Figure 3B). A hydrophilicity plot of 22 DED sequences (Peter *et al.*, 1997) revealed that all DED contained two characteristic hydrophilic stretches. Comparison of the putative DED of DEDD with the DED of all these proteins showed that the locations of the two hydrophilic regions were most similar to the ones found in the DED of FADD and the first DED of caspase-8 (Figure 3C and data not shown). Independent alignments using the Lipman–Pearson method revealed homologies to DNA binding proteins at the C terminus such as SAF-B, a SAR binding protein (Renz and Fackelmayer, 1996), and three histones, H1, H2A and H4 within the center of the histone fold (Luger and Richmond, 1998) (Figure 3D).

DEDD induces apoptosis through its DED

To determine whether the putative DED in DEDD was functionally active, two DEDD truncation mutants were generated (Figure 4A). N-DEDD spans positions 1–114, including the DED and the adjacent NLS1. C-DEDD spans position 109–318 containing the proline-rich region, NLS2 and the C-terminal half of DEDD. To minimize the risk of interference with the functions of these proteins, N-DEDD was tagged with a FLAG peptide at its C terminus, whereas C-DEDD was constructed with an N-terminal FLAG tag. To test whether DEDD could activate the apoptosis machinery through its DED, 293T

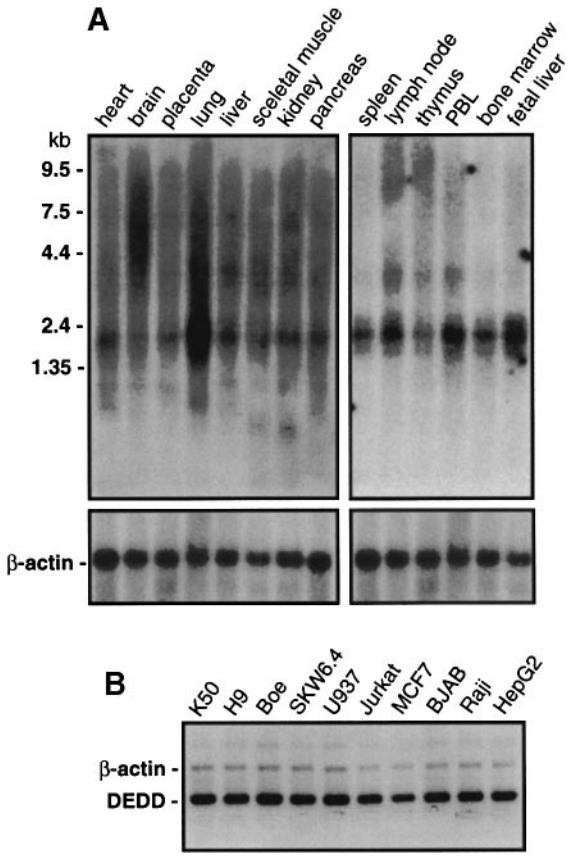


Fig. 2. mRNA distribution of DEDD in several tissues and cell lines. (**A**) Northern blot analysis with a poly(A)$^+$ RNA hybridized membrane probed with a DEDD oligonucleotide encoding the N-terminal DED-containing domain (N-DEDD) The individual tissues analyzed are indicated above the lanes. β-actin is shown as a loading control. (**B**) RT–PCR of DEDD from several cell lines of lymphoid and non-lymphoid origin. β-actin primers were used as an internal control.

Fig. 1. Identification of DEDD as a new death effector domain (DED)-containing protein with high homology between mouse and man. (**A**) A database search for new DED-containing proteins revealed a mouse EST clone (DDBJ/EMBL/GenBank accession number: Aa124451) with a DED homologous to the DED of FADD and caspase-8. Identification of overlapping EST nucleotide sequences resulted in the determination of the full-length DEDD protein sequence. For the human ESTs, the tissue from which each EST clone was derived is given underneath each clone. All mouse EST sequences (except Aa163060) were derived from mouse embryos. For each EST clone, only the DEDD coding areas are shown and numbers indicate amino acids as found in the complete DEDD protein sequence. Stippled lines indicate corresponding sequences located outside the ORF. Steps in lines represent frameshifts caused by missequenced bases found in the EST database. (**B**) Comparison of murine and human DEDD on DNA as well as on protein level. Differences in nucleotides are shown by open boxes and differences on the protein level by closed boxes. Positions of two putative nuclear localization signals are shown by lines.

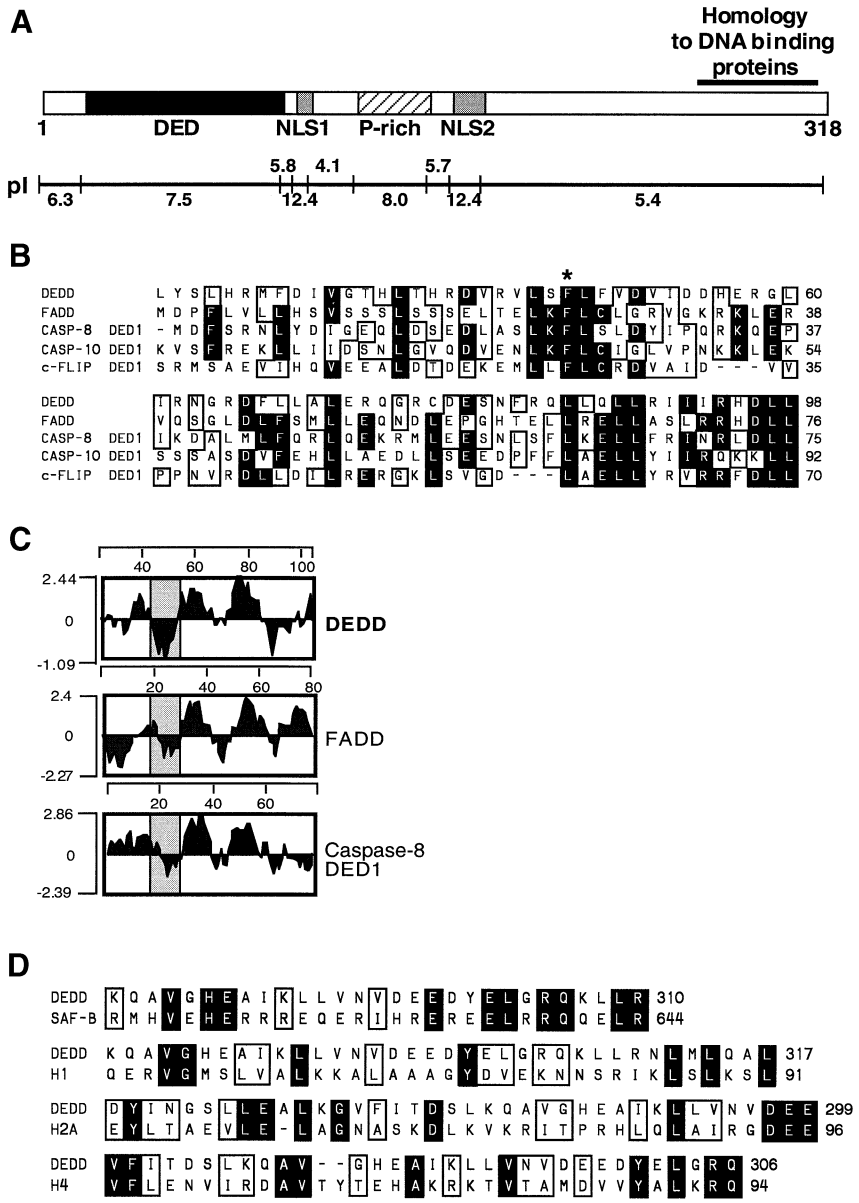


Fig. 3. Structural organization of the DEDD polypeptide. (**A**) Schematic representation of the subdomains of DEDD. DED, death effector domain; NLS, nuclear localization signal; P-rich, proline-rich region. The isoelectric points (pI) of the several subdomains are indicated below. (**B**) The DED of DEDD contains an area of significant homology to the DED of FADD (18.4% identity, 48.7% conservation), to the first DED of caspase-8 (CASP-8) (21.0% identity, 40.7% conservation), to the first DED of caspase-10 (CASP-10) (18.4% identity, 42.3% conservation), and to the first DED of c-FLIP (19.7% identity, 41.7% conservation). Identical amino acid residues are depicted in black boxes; conservative exchanges are indicated by white boxes. The asterisk marks the position of the conserved phenylalanine residue important for DED/DED interaction-mediated cytotoxicity (Eberstadt *et al.*, 1998). (**C**) Hydrophilicity plot of the DED of DEDD, FADD and caspase-8. The location of a recently identified hydrophobic patch containing the conserved phenylalanine residue essential for DED-mediated interactions (Eberstadt *et al.*, 1998) is labeled by a shaded box. (**D**) The C terminus of DEDD shows homology to histone H1 (36 aa, 19.4% identity, 55.5% conservation), H2A (38 aa, 25.6% identity, 52.6% conservation), H4 (34 aa, 28.6% identity, 55.9% conservation), and to the SAR-binding protein SAF-B (29 aa, 34.5% identity, 44.8% conservation).

cells were transiently transfected with an expression vector encoding DEDD and nuclear fragmentation was quantified. Wild-type DEDD weakly induced apoptosis (Figure 4B), the degree of apoptosis being comparable with that induced by caspase-8, but lower when compared with FADD. Removing 60% of the C terminus of DEDD resulted in a protein which was much more potent in induction of apoptosis than wild-type DEDD (N-DEDD in Figure 4B), demonstrating that the DED of DEDD is functional and that the C-terminal half of DEDD has anti-apoptotic activity. Both wild-type DEDD- and N-DEDD-induced apoptosis could be blocked by co-expression of the serpin caspase inhibitor crmA, indicating that DEDD-induced apoptosis requires caspase activation, as do other DED-containing apoptosis signaling molecules. To test for functional association of DEDD with FADD or caspase-8, suboptimal non-cytotoxic concentrations of FADD or caspase-8 were co-transfected with suboptimal concentrations of the DEDD deletion mutants (Figure 4C). Under these conditions, FADD could enhance DEDD-induced apoptosis only in the absence of the C-terminal part of DEDD, i.e. N-DEDD was much more cytotoxic in the

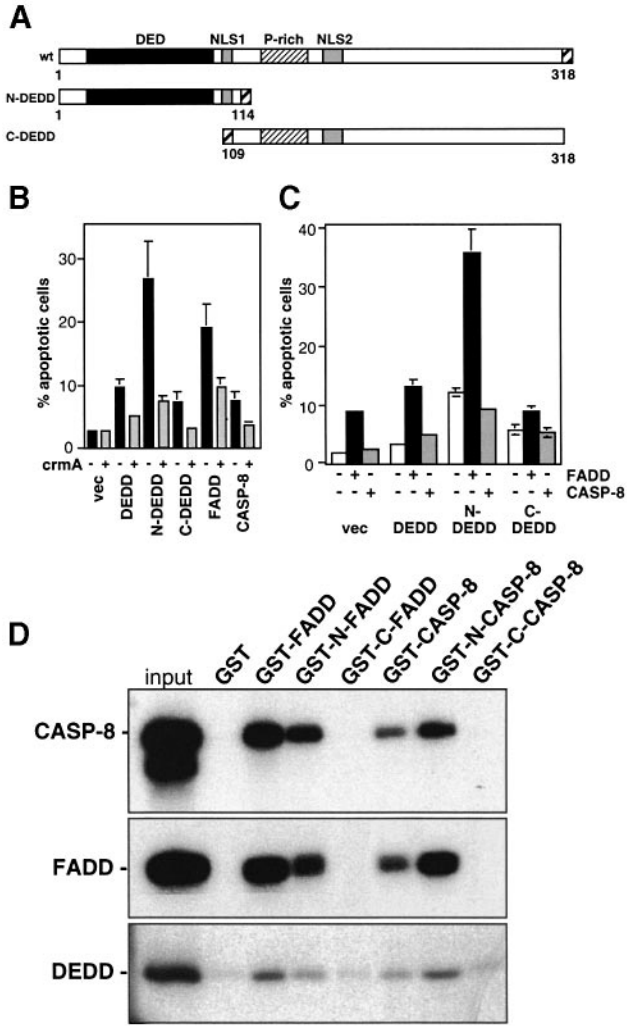


Fig. 4. The DED in DEDD is functionally active. (**A**) Scheme showing the structure of N-DEDD and C-DEDD. A FLAG tag was added to the 3′ end of wild-type DEDD and N-DEDD or to the 5′ end of C-DEDD. (**B**) Induction of apoptosis by transient transfection of DEDD mutants into 293T cells. The amount of DNA used for each DEDD construct was 3 μg and for the pFM91-crmA plasmid was 4 μg. All DEDD constructs had a 3′FLAG tag. Only C-DEDD was tagged at the 5′ end to allow potential interaction with the C terminus of C-DEDD. All experiments were performed with 3′FLAG-tagged DEDD and N-DEDD. 5′FLAG-tagged DEDD or N-DEDD were also tested and found to be much less potent in induction of apoptosis (data not shown). Untagged DEDD behaved exactly like 3′FLAG-DEDD, excluding the possibility that the 3′FLAG tag interfered with apoptosis-inducing activity of DEDD. DNA fragmentation was determined as described in Materials and methods. Means (± SD) of three independent experiments are shown. (**C**) Enhancement of N-DEDD-induced apoptosis by FADD. Suboptimal, non-cytotoxic amounts (0.5 μg) of pcDNA3-3′FLAG-DEDD, pcDNA3-3′FLAG-N-DEDD or pcDNA3-5′FLAG-C-DEDD were co-transfected with suboptimal DNA concentrations (0.3 μg) of either pcDNA3-AU1-FADD or pcDNA3-caspase-8. DNA fragmentation was determined as described in Materials and methods. Means (± SD) of three independent experiments are shown. (**D**) DEDD interacts with DED containing GST fusion proteins of caspase-8 and FADD. Several GST fusion proteins as indicated were incubated with either *in vitro*-translated [^{35}S]caspase-8 and [^{35}S]FADD or [^{35}S]DEDD. The precipitates were subjected to 12% PAGE and subsequently analyzed by autoradiography. The migration positions of caspase-8 (CASP-8), FADD and DEDD are indicated.

presence of small amounts of FADD when compared with wild-type or C-DEDD. Co-transfection of caspase-8 did not have any effect, even at higher concentrations (data not shown). The observation that C-DEDD-mediated apoptosis could not be enhanced by FADD suggests that the DED in DEDD is required for induction of apoptosis.

DEDD binds to FADD and caspase-8 through DED/DED interaction

The cooperative effect of FADD to enhance N-DEDD-mediated apoptosis suggested that DEDD was involved in death receptor signaling through DED/DED interactions. To test whether DEDD interacts with FADD and/or caspase-8, precipitation experiments were performed using glutathione *S*-transferase (GST)–FADD and GST–caspase-8 fusion constructs incubated with ^{35}S-labeled *in vitro*-translated FADD, caspase-8 or DEDD. [^{35}S]DEDD bound only to the DED containing GST fusion proteins, i.e. GST–FADD, GST–N-FADD, GST–caspase-8 and GST–N-caspase-8 (Figure 4D). None of the *in vitro*-translated proteins bound to GST alone, to GST–C-FADD or to GST–C-caspase-8, demonstrating that DEDD binds to the DED of FADD and caspase-8 *in vitro*. In summary, the data suggest that DEDD is functional, binds to other DED-containing proteins like caspase-8 or FADD, induces apoptosis when expressed in 293T cells, and that this apoptosis can be enhanced by FADD. FADD may therefore be a physiological interaction partner for DEDD.

DEDD is located in nucleoli

The presence of two putative NLS in DEDD suggests that it could be localized within the nucleus. To test this, pcDNA3-DEDD was transiently transfected into 293T cells and the subcellular localization of DEDD determined by Western blotting using an affinity-purified DEDD-specific anti-peptide rabbit antiserum after fractionation into nuclear, cytoplasmic and microsomal fractions. As shown in Figure 5A, DEDD was found only in the nuclear fraction of cells transfected with the DEDD expression vector. Detection of this band was abolished by preincubation of the anti-DEDD antibody with the peptide used for antibody generation (Figure 5A, lane 7). Consistent with their cytosolic location, both FADD and caspase-8 were found predominantly in the cytoplasm.

To demonstrate the nuclear localization of DEDD more directly, a FLAG-tagged DEDD was transfected into 293T cells and visualized by immunofluorescence microscopy (Figure 5B). These experiments revealed that DEDD was not uniformly distributed within the nucleoplasm, but rather accumulated in distinct globular structures which appeared to be nucleoli. To prove this, the cells were co-stained with both the anti-FLAG mAb and an antibody against the upstream binding factor (UBF), a basal factor for RNA polymerase I transcription, which is exclusively located in nucleoli (Chan *et al.*, 1991) (Figure 5C, center panel). Superimposition of both stainings revealed that DEDD co-localized precisely with UBF (Figure 5C, right panel). Thus, DEDD overexpressed in 293T cells is found exclusively in nucleoli.

In vivo, DEDD is localized in the cytoplasm and translocates to the nucleus upon stimulation of CD95

The experiments on the intracellular localization of DEDD described so far have been performed in cells overexpress-

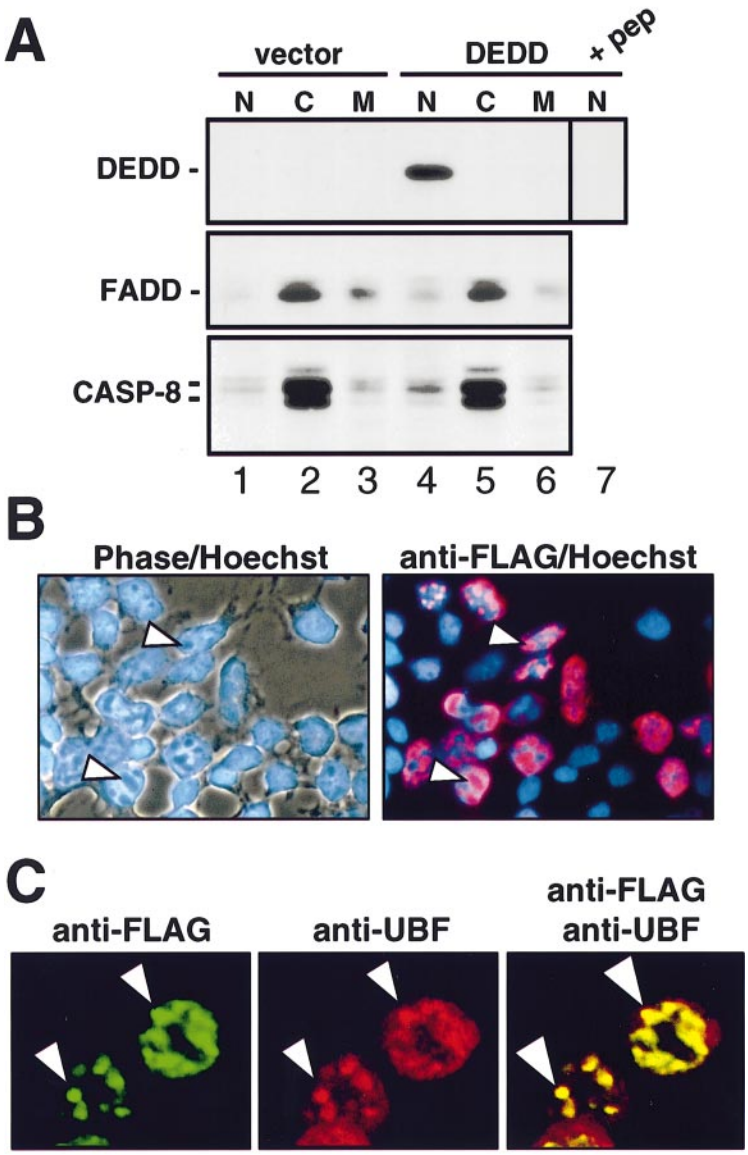


Fig. 5. Subcellular distribution of DEDD. (**A**) DEDD is localized in the nuclear compartment. 293T cells transfected either with 5 μg pcDNA3 or pcDNA3-DEDD were fractionated into nuclei (N), cytoplasm (C) and membrane fractions (M) and the distribution of DEDD was analyzed by Western blotting using an affinity-purified polyclonal anti-DEDD antibody (top panel), anti-FADD mAb (center panel) and anti-caspase-8 mAb (bottom panel). Lane 7, nuclear extracts from pcDNA3-DEDD-transfected 293T cells developed with the polyclonal anti-DEDD antibody preincubated with the DEDD peptide used for immunization. The nuclear localization of DEDD was additionally confirmed by transfecting a GFP–DEDD fusion protein into 293T cells. GFP–DEDD was again found only in the nuclei (data not shown). (**B**) 293T cells transfected with 3 μg pcDNA3-5′FLAG-DEDD were subjected to immunofluorescence microscopy using an anti-FLAG antibody (red fluorescence, right panel). The transfected cells were additionally stained with the DNA-intercalating dye Hoechst 33258 (blue fluorescence) marking size and form of the nuclei. Globular structures within nuclei intensively stained by the anti-FLAG mAb also visible in phase contrast are labeled by arrow heads. (**C**) Co-localization of DEDD with UBF. 293T cells transfected with 3 μg pcDNA3-3′FLAG-DEDD were stained for DEDD using an anti-FLAG antibody with a FITC-labeled secondary antibody (green fluorescence, left panel) and for the nucleolar transcription factor UBF using a human anti-UBF serum and a Texas red-coupled secondary antibody (red fluorescence, center panel). The overlay of both images is shown in the right panel. The localizations of DEDD and UBF in the single fluorescence and in the overlay are indicated by arrowheads.

ing DEDD. To investigate the intracellular localization of endogenous DEDD, we determined its distribution in a number of lymphoid cells using the affinity-purified anti-DEDD rabbit antibody. In all cells tested, a specific protein of 37 kDa was detected with the same electrophoretic mobility as DEDD overexpressed in 293T cells (Figure 6A). In contrast to the latter, endogenous DEDD was found exclusively in the cytoplasm. Since overexpression of DEDD and *in vitro* association with FADD and caspase-8 indicated that DEDD was involved in death receptor signaling, we treated Jurkat and CEM T cells with anti-APO-1 to induce apoptosis and analyzed the localization of DEDD at different times after stimulation (Figure 6B and C). In both cell lines DEDD appeared in the nuclear fraction first detectable after 10 min. Specificity of the detection of DEDD in Western blot was again established by competition with the DEDD peptide (Figure 6B, bottom panel). Consistent with the overexpression experiment in the 293T cells, *in vivo* translocation of DEDD to the nucleus required activation of caspases as it could be partially blocked by pretreating CEM cells with the broad-spectrum caspase inhibitor zVAD-fmk (Figure 6C, lanes

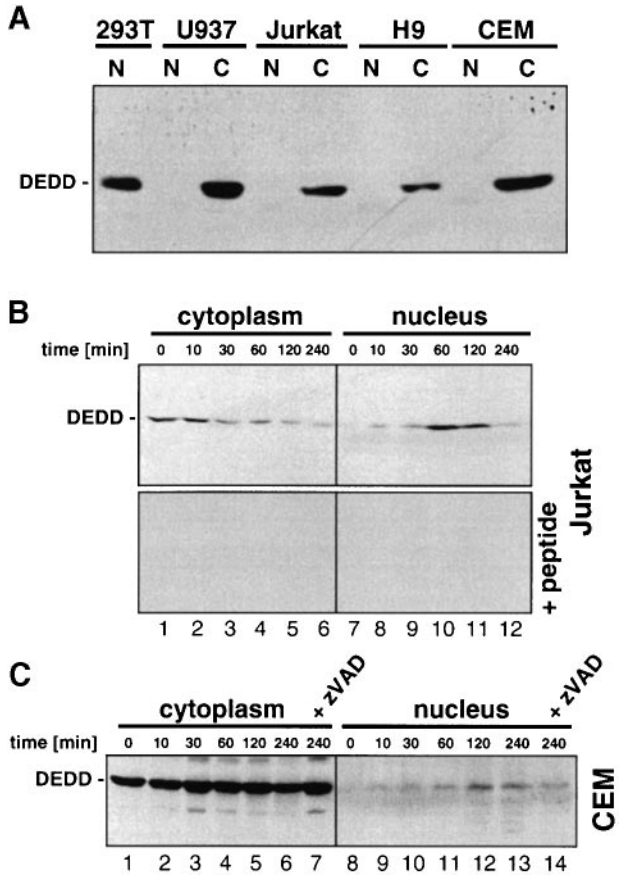


Fig. 6. *In vivo* localization of DEDD. (**A**) Expression of endogenous DEDD in the cytoplasm of lymphoid cell lines. Anti-DEDD Western blot of nuclear (N) and cytoplasmic (C) fractions from 5×10^6 cells. The nuclear compartment of 10^5 293T cells transfected with pcDNA3-DEDD is shown as a migration control. (**B**) Translocation of DEDD from the cytosol to nucleus during CD95-mediated apoptosis. Jurkat cells (5×10^6) were treated with anti-APO-1 for the indicated time periods, subjected to subcellular fractionation, and the Western blot developed with the polyclonal anti-DEDD antibody (upper panel) or preincubated with the DEDD peptide (bottom panel). The migration position of DEDD is indicated. (**C**) CEM cells (5×10^6) treated with anti-APO-1 for different time points were analyzed as in (B). Samples in lanes 7 and 14 were preincubated with zVAD-fmk (+ zVAD). The decrease of the total amount of DEDD in Jurkat and CEM cells after 240 min of anti-APO-1 treatment is due to a general loss of proteins at this advanced stage of apoptosis.

7 and 14). The data show that during CD95-mediated apoptosis, up to 80% of cytoplasmic DEDD (in the Jurkat cells) translocates to the nucleus. Quantitative Western blots revealed that DEDD is an abundant protein, being present in ~200 000 copies in the cytoplasm of Jurkat cells. Hence, up to 160 000 molecules of DEDD may accumulate in the nucleus of these cells during CD95-mediated apoptosis.

Translocation of DEDD to the nucleus requires caspase activation

The results presented so far indicate that DEDD could translocate to the nucleus both *in vivo* and in an overexpression system. To test whether the quantitative translocation of overexpressed DEDD was linked to its apoptotic activity, the effect of blocking apoptosis by co-transfection of crmA (Figure 4B) on the translocation of DEDD was tested. Upon co-transfection of DEDD and crmA, in addition to cells exhibiting the nuclear staining (Figure 7A, arrowhead in top row), ~50% of the transfected cells showed a pronounced cytoplasmic distribution of DEDD (Figure 7A, arrows in top row). This effect of crmA suggested again that apoptosis and caspase activation were needed for translocation of DEDD to the nucleus. Since overexpression of DEDD resulted in weak apoptosis, DEDD itself may have provided the necessary apoptosis signal facilitating its translocation to the nucleus. When 293T cells were transfected with DEDD and crmA, or treated with zVAD-fmk, a less efficient translocation of DEDD into the nucleus was observed (Figure 7B, top row). Both N-DEDD and C-DEDD, each of which contain one NLS (NLS1 or NLS2, respectively), were still found in the nucleus as revealed by anti-FLAG immunofluorescence (Figure 7A, middle and bottom rows) and subcellular fractionation (Figure 7B, middle and bottom rows). However, blocking of apoptosis with crmA or zVAD-fmk only affected the localization of C-DEDD, resulting in a pronounced cytoplasmic distribution in immunofluorescence microscopy (Figure 7A, bottom row) and subcellular fractionation (Figure 7B, lower panel). The nuclear localization of N-DEDD did not change under apoptosis-inhibiting conditions (Figure 7A and B, middle rows). These data indicate that NLS2 is inducible and requires activation of caspases. Interestingly, the subnuclear localization of N-DEDD and C-DEDD appeared different. Similar to wild-type DEDD, N-DEDD containing NLS1 was localized in nucleoli, whereas C-DEDD having NLS2 showed a more diffuse nuclear localization (Figure 7A, left column, center and bottom panel). The data suggest that both NLS can individually direct DEDD to the nucleus. However, in this overexpression system NLS1 is constitutively active and mainly responsible for the nucleolar localization of DEDD, whereas NLS2 requires activation of caspases to be functional. The contribution of both NLS in DEDD to nuclear and nucleolar localization were confirmed by using NLS1, NLS2 or NLS1/2 deletion mutants in immunofluorescence microscopy (data not shown).

DEDD associates with FADD during CD95-mediated apoptosis in vivo

Translocation of overexpressed DEDD to the nucleus could be partially prevented by blocking activation of caspases (Figure 7). Since DEDD was found to associate with FADD and caspase-8 *in vitro*, we tested whether under conditions with significant amounts of DEDD in the cytoplasm an association with endogenous FADD and/or caspase-8 could be found *in vivo*. To this end, DEDD was expressed in 293T cells in the presence of crmA. FADD and caspase-8 were immunoprecipitated with specific monoclonal antibodies and the amount of co-immunoprecipitated DEDD was determined by Western blot analysis using the anti-DEDD antibody (Figure 8A). DEDD was found to be weakly associated with caspase-8 and much stronger with FADD, whereas DEDD was not found in the immunoprecipitate using the control mAb FII23. These data pointed to FADD and/or caspase-8 as potential interaction partners of DEDD during CD95-mediated apoptosis *in vivo*. Jurkat cells were therefore stimulated with anti-APO-1 for different periods of time. Subsequently, caspase-8 and FADD were immunoprecipitated and association of DEDD was again determined by

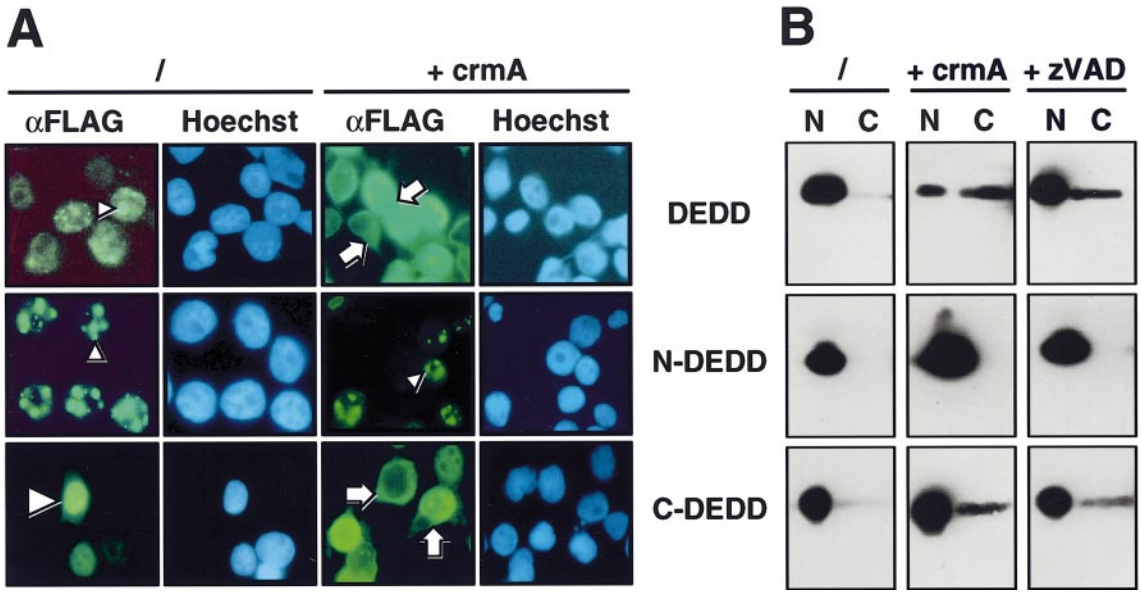


Fig. 7. Caspase inhibition prevents translocation of DEDD into the nucleus. (**A**) Analysis of the localization of DEDD and DEDD deletion mutants by immunofluorescence microscopy. 293T cells were transfected with 3 μg pcDNA3-3′FLAG-DEDD, pcDNA3-3′FLAG-N-DEDD and pcDNA3-5′FLAG-C-DEDD either alone or together with 4 μg pFM91-crmA, respectively, and DEDD was analyzed by immunostaining with an anti-FLAG antibody and a FITC-labeled secondary antibody. Nucleolar localization is indicated by small arrowheads, nucleoplasmic staining by large arrowheads, and cytoplasmic distribution by arrows. (**B**) Localization of wild-type DEDD, N-DEDD and C-DEDD. Nuclear (N) and cytoplasmic (C) compartments of the transfected 293T cells co-transfected with DEDD and crmA [see (A)] or transfected with DEDD in the presence of zVAD-fmk were subjected to anti-FLAG Western blotting.

Western blot analysis (Figure 8B). Under these conditions no association of DEDD with caspase-8 was found, whereas FADD associated with DEDD in a stimulation-dependent manner. First association of FADD with DEDD was detectable 10 min after CD95 triggering, correlating kinetically with the *in vivo* translocation of DEDD from cytoplasm to nucleus (Figure 6B). These data confirm that DEDD carries a functional DED by which it associates physiologically with FADD during CD95-mediated apoptosis.

DEDD is a DNA- and nucleosome-binding protein that inhibits rDNA transcription

The sequence homology of the C-terminal part of DEDD to histones (Figure 3D) suggests that DEDD may bind to DNA. To address this issue, GST–DEDD was expressed in *Escherichia coli* and the affinity-purified protein tested in electrophoretic mobility shift assays. As shown in Figure 9A, incubation of GST–DEDD with DNA resulted in the formation of large aggregates which did not penetrate agarose gels. This complex formation was not observed with GST alone or with a GST–FADD fusion protein (Figure 9A, lanes 2 and 3). DEDD/DNA complexes were formed at high salt concentrations (up to 2 M NaCl; Figure 9A, lane 7), demonstrating the high affinity of DEDD binding to DNA. As could be expected from the sequence homology to histones, DEDD binds non-specifically to DNA. Any DNA tested, i.e. phage λ DNA (Figure 9A), herring sperm DNA or different oligonucleotides ranging in length between 22 and 64 bp (data not shown) was bound, yielding large aggregates which accumulated on top of the gel.

Both the DNA-binding properties and the nucleolar localization of DEDD suggest that it may interfere with important nucleolar function(s). We therefore investigated whether DEDD would inhibit rDNA transcription. Since DEDD aggregates DNA, we decided to use a more physiological template, namely chromatin. First, we tested whether DEDD would also aggregate nucleosomal DNA. In the experiment shown in Figure 9B, a 248 bp fragment encompassing the rDNA promoter was assembled into mononucleosomes, incubated with GST–DEDD, and binding to both free DNA and mononucleosomal DNA was compared in electrophoretic mobility shift assays. DEDD, but not FADD, bound both free DNA and mononucleosomal DNA (Figure 9B, lanes 3 and 6). In contrast to free DNA, binding of DEDD to mononucleosomes yielded a defined complex with retarded electrophoretic mobility. To compare the affinity of DEDD to free and mononucleosomal DNA, a titration was performed (Figure 9C). Significantly, the amount of DEDD required to shift the labeled probe was one order of magnitude higher on nucleosomes as compared with naked DNA. At higher DNA:protein ratios, a second defined complex was formed. A defined complex was also formed on minimal nucleosomes that were reconstituted on a 146 bp DNA fragment, and therefore, did not contain free DNA ends (data not shown).

The higher specificity of binding of DEDD to nucleosomal compared with free DNA encouraged us to test the effect of DEDD on transcription of ribosomal gene templates assembled into chromatin. For this, an artificial ribosomal minigene construct was used ($pMrT_2$) which represents a fusion between a 5′-terminal murine rDNA fragment containing the rDNA promoter and a 3′-terminal rDNA fragment containing a terminator element (Längst *et al.*, 1998). The plasmid DNA was assembled into chromatin with extracts from early *Drosophila* embryos and used as a template for *in vitro* transcription. As has been shown previously (Längst *et al.*, 1997), in the absence of the transcription termination factor TTF-I, transcription

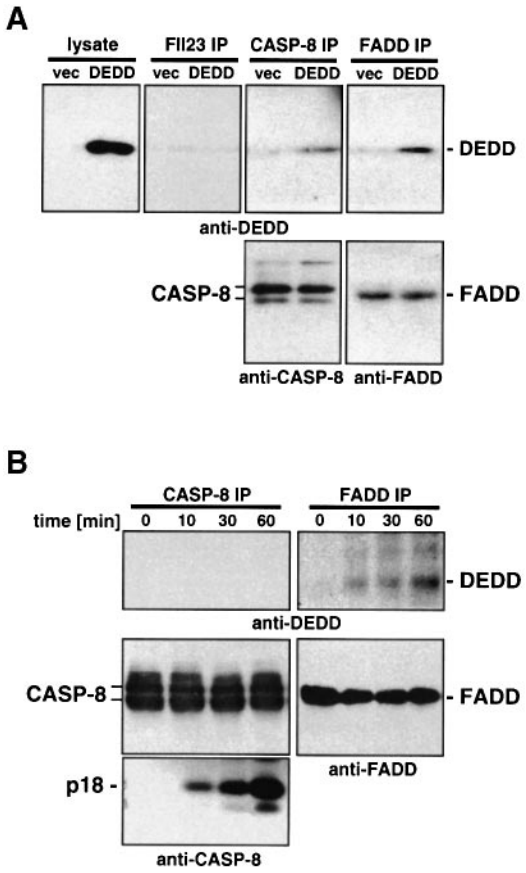


Fig. 8. *In vivo* association of DEDD with FADD. (**A**) 293T cells were transfected with 8 μg pFM91-crmA together with either 6 μg pcDNA3 (vec) or 6 μg pcDNA3-3′FLAG-DEDD (DEDD). Cells were lysed and either directly subjected to a Western blot analysis (lysate) or proteins were first immunoprecipitated with control antibody (FII23 IP), anti-caspase-8 C1 (CASP-8 IP) or anti-FADD 1C4 (FADD IP). Immunoprecipitates were subjected to anti-DEDD Western blot analysis (upper panels). A control Western blot was done using anti-CASP-8 (C15) or anti-FADD (1B5) to ensure equal levels of immunoprecipitated proteins (bottom panels). (**B**) Jurkat cells were treated with 2 μg/ml anti-APO-1 for the indicated periods of time and subsequently lysed. Caspase-8 and FADD were immunoprecipitated and Western blot analyses performed as described in (A). The migration positions of caspase-8 (CASP-8), the active caspase-8 subunit p18, FADD and DEDD are labeled.

on reconstituted chromatin templates was repressed (Figure 9D, lane 1). Addition of TTF-I relieved repression and promoted synthesis of 180 nt RNA, i.e. transcripts that were initiated at the transcription start site and stopped at the terminator (lane 2). Significantly, transcription was abolished in the presence of low amounts of GST–DEDD (lane 3), whereas 24-fold higher amounts of GST–FADD had no effect (lane 4). Taken together, DEDD is a novel death effector domain protein that contains two NLS. Upon triggering of CD95 (APO-1/Fas), DEDD translocates to the nucleus where it localizes to the nucleolus. DEDD has a very strong histone-like DNA-binding activity and it inhibits transcription of rDNA in an *in vitro* transcription assay with reconstituted chromatin.

Discussion

DEDD contains a functional death effector domain

Overexpression of DED-containing proteins in 293T cells often results in induction of apoptosis, probably due to engagement of other DED-containing proteins such as FADD or by direct binding to DED-containing caspases such as caspase-8 (Eberstadt *et al.*, 1998). Also, DEDD weakly induced apoptosis and it was therefore impossible to generate stable DEDD transfectants. The C-terminal part of DEDD (C-DEDD) alone weakly induced apoptosis. However, N-DEDD which contained only the DED and the first NLS was more effective in inducting apoptosis than full-length DEDD or C-DEDD. Furthermore, N-DEDD-induced apoptosis could be strongly enhanced by co-expression of FADD. This suggests that the C terminus of DEDD somehow hindered binding of DEDD to other DED proteins, thus exhibiting an anti-apoptotic function. More importantly, these data suggested that FADD is a physiological interaction partner of DEDD, an assumption which was confirmed by *in vivo* immunoprecipitation experiments using Jurkat T cells. FADD, but not caspase-8, associated with DEDD upon triggering of CD95.

DEDD is a highly conserved protein with strong DNA-binding activity

Human and mouse DEDD are almost identical (98.7% identity), this level of conservation being found throughout the molecule. In particular, the C-terminal half of DEDD and the DED are completely identical between human and mouse, such extreme conservation being much higher than that usually found when comparing DEDs of other DED-containing proteins from man or mouse (70.7% identity for the DED 1 of caspase-8; 81.8% identity for the DED of FADD).

Only proteins that act at intracellular key control points have a similarly high conservation between man and mouse. These proteins are found in the respiratory chain within mitochondria (e.g. cytochrome *c* = 91.3% identity; cytochrome *c* oxidase subunit 1= 90.4%), in ribosomes (e.g. L7 = 95.9%; L12 = 98.8%; L18 = 90.4%; L19 = 99.5%; L28 = 97.8%; S2 = 98.2%; S16 = 97.3%) or among histones (H3 = 100%, H4 = 100%). The degree of identity of DEDD (98.7%) between human and mouse suggests that, in addition to its role in coupling to the apoptosis machinery via DED-mediated interactions, DEDD may have other crucial functions that require such high conservation throughout evolution. These functions seem to be important for all cells since DEDD is expressed ubiquitously. This could already be concluded from 23 DEDD EST sequences found in the initial database searches obtained from various tissues, suggesting that DEDD is very abundant on the mRNA level.

Consistent with the presence of two NLS and sequence homology to a SAR binding protein and histones, DEDD binds DNA with very high affinity and no apparent sequence specificity. Upon incubation with DEDD, large DNA aggregates were formed which did not penetrate the gels. The formation of these DEDD/DNA complexes suggests that DEDD may have more than one DNA-binding domain and thus crosslinks DNA; alternatively, DNA could induce aggregation of DEDD. N-DEDD also bound DNA (data not shown), indicating that the DED was directly involved in this DNA binding, and consistent with the fact that the DED of mouse and human DEDD are completely identical.

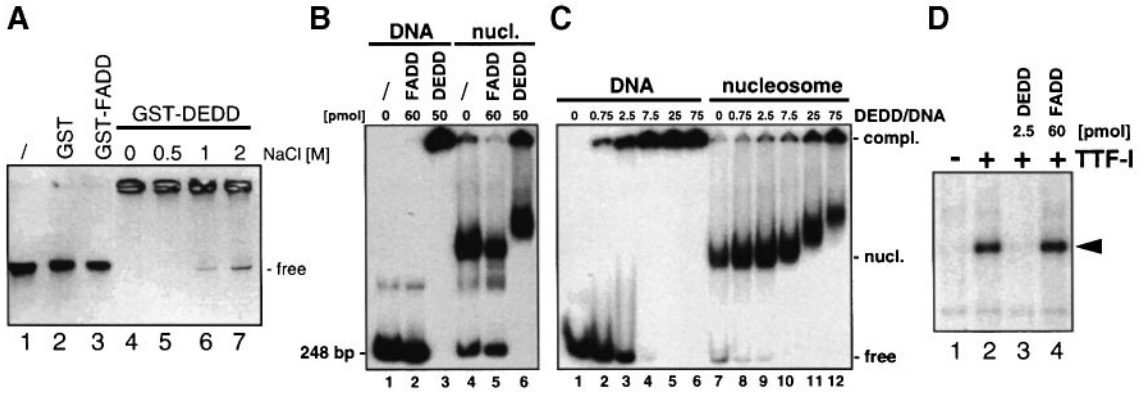


Fig. 9. DEDD forms complexes with both DNA and nucleosomes and inhibits rDNA transcription. (**A**) High-affinity binding of DEDD to DNA. 1 μg of phage λ DNA was incubated for 10 min at room temperature with 1 μg of GST, GST–DEDD or GST–FADD in the presence of increasing NaCl concentrations, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining. Lane 1 shows the mobility of λ DNA without addition of protein. The migration position of free DNA (free) is shown. (**B**) DEDD binds to nucleosomal DNA. A 248 bp 5′ end-labeled DNA fragment containing murine rDNA promoter sequences (positions –232 to +16) was assembled into mononucleosomes. 80 ng of free DNA (lanes 1–3) and nucleosomal DNA (lanes 4–6) were incubated with the indicated amounts of GST–FADD (lanes 2 and 5) or GST–DEDD (lanes 3 and 6). DNA–protein complexes were analyzed by the electrophoretic mobility shift assay. (**C**) Titration of DEDD into free and mononucleosomal DNA. 50 ng of free DNA (lanes 1–6) and nucleosomal DNA (lanes 7–12) were incubated with GST–DEDD at the indicated DEDD/DNA ratios. DNA–protein complexes were analyzed by the electrophoretic mobility shift assay. Migration positions of free and complexed (compl.) DNA and of mononucleosomes (nucl.) are labeled. (**D**) *In vitro* transcription on nucleosomal rDNA. The rDNA minigene $pMrT_2$ was assembled into chromatin in the absence (lane 1) or presence (lanes 2–4) of the transcription termination factor TTF-I. 20 ng of nucleosomal DNA were used as template in transcription reactions containing partially purified murine Pol I and transcription factors. GST–FADD or GST–DEDD were added in the indicated amounts. Specific transcripts (arrowhead) were separated on 4.5% polyacrylamide gels and visualized by autoradiography.

DEDD contains two functional NLS that direct DEDD to the nucleolus and may affect nucleolar functions

Two putative NLS sequences, one classical hexapeptide NLS and one bipartite NLS, were identified in the sequence of DEDD. There are a number of proteins, mostly transcription factors, that contain two NLS such as certain HMG box-containing proteins (e.g. SRY or SOX9; Sudbeck and Scherer, 1997) or the family of Krüppel-like factors (transcription factors with a Krüppel-like zinc finger motif) (Shields and Yang, 1997). Some protein families even contain multiple NLS, such as the estrogen and progesterone receptors (Ylikomi *et al.*, 1992). Similar to the situation in DEDD, one NLS often potentiates or complements the function of another. Our data suggest that NLS2 directs DEDD into the nucleus, whereas NLS1 appears to be involved in targeting DEDD to the nucleolus. In support of this view, the mutant protein C-DEDD bearing only the second NLS and ΔNLS1-DEDD exhibited a nucleoplasmic localization, whereas N-DEDD and ΔNLS2-DEDD were found only in the nucleolus.

DEDD inhibits transcription of rDNA

The nucleolar localization of DEDD suggests that it may affect some important nucleolar functions, and therefore interferes with ribosome biosynthesis. The nucleolus, i.e. the organelle where transcription, modification, processing and assembly of pre-RNA take place, may be a preferred intracellular target for apoptotic events, because inhibition of any of these events would prevent the formation of ribosomes and consequently block cellular biosynthetic activities. Although induction of apoptosis by CD95 is a very rapid process, a large number of caspase targets are key components for several cellular activities, such as DNA replication, transcription, RNA processing and translation—all processes that may occur late after the onset of CD95-mediated apoptosis. In support of this view, the DNA replication machinery contains a target of caspase-3 (Rheaume *et al.*, 1997; Song *et al.*, 1997; Ubeda and Habener, 1997). Cleavage of RF-C140, a crucial part of the DNA replication complex, during CD95-mediated apoptosis results in cell cycle arrest. The protein synthesis machinery is also a target for degradation. Shortly after triggering of CD95, the polysomes disintegrate, losing their biosynthetic activity (Zhou *et al.*, 1998). In addition, during many forms of apoptosis, ribosomes become functionally inactivated by specific degradation of the 28S rRNA within the ribosome (Houge *et al.*, 1995). This would make cellular rRNA synthesis the rate-limiting step of protein synthetic activity, regardless of the long half-life of ribosomes. It is therefore conceivable that the transcription of ribosomal genes in the nucleoli is also downregulated. A report demonstrating proteolytic degradation of UBF, a critical factor in the regulation of rDNA transcription, early (after 2 h) during CD95-mediated apoptosis supports the idea that inhibition of transcription of rDNA is an important step in the process of CD95-mediated apoptosis (Casiano and Tan, 1996; Casiano *et al.*, 1996, 1998). As a first step to find out which nucleolar function is affected by DEDD, we assayed the effect of recombinant DEDD on rDNA transcription *in vitro*. Since DEDD—like histone H1—binds non-specifically to DNA and represses both RNA polymerase I and II transcription (Croston *et al.*, 1991; Kuhn and Grummt, 1992), we tested its effect on transcription by RNA polymerase I on a chromatin template. The reconstituted transcription system used contains an artificial ribosomal minigene that has been assembled into chromatin, both partially purified Pol I and Pol I-specific transcription initiation factors, and recombinant TTF-I, the Pol I-specific transcription termination factor. Previous studies have established that, in this system, faithfully initiated and terminated transcripts are synthesized (Längst *et al.*, 1997, 1998). Consistent with its nucleolar localiz-

ation, low amounts of DEDD (below those required to shift the electrophoretic mobility of mononucleosomes) strongly inhibited rDNA transcription. In contrast, 24-fold higher levels of FADD had no effect. Whether or not DEDD-mediated transcriptional repression is specific for ribosomal gene transcription by Pol I remains to be investigated. Nevertheless, the availability of this *in vitro* system will facilitate studies concerning the molecular mechanism underlying repression of rRNA synthesis by DEDD.

A model for DEDD activation and translocation to the nucleus

Overexpression of DEDD resulted in weak induction of apoptosis, presumably by binding to FADD through DED/DED interaction. In contrast to FADD and caspase-8, DEDD was not found to be involved in the formation of the CD95 DISC (data not shown), suggesting that DEDD may exert its function downstream of these receptor proximal events. Overexpressed DEDD was found exclusively in the nuclear fraction, whereas endogenous DEDD in non-apoptotic cells was found only in the cytoplasm. Translocation into the nucleus could be significantly inhibited by co-expression of crmA or treatment with the caspase peptide inhibitor zVAD-fmk, indicating that caspase activation was required for this process. However, association of DEDD with FADD did not require activation of caspases since it was detected in the presence of overexpressed crmA. This association therefore appears to occur either independently or upstream of the activation of caspases.

On the basis of these data, we propose the following model for activation of DEDD. In non-apoptotic cells, DEDD is localized in the cytoplasm. We suggest the existence of an inhibitor that binds to the C terminus of DEDD and thus masks NLS2. After induction of apoptosis through CD95, the caspase cascade is activated, which leads to cleavage of this molecule and results in its degradation and/or its release from DEDD. The concept of a caspase cleaved inhibitor was introduced recently. An endonuclease, CAD, was found to be complexed to an inhibitor, ICAD, likely preventing CAD from entering the nucleus (Enari *et al.*, 1998; Sakahira *et al.*, 1998). ICAD is a substrate of caspase-3, and cleavage of ICAD resulted in liberation of the active endonuclease CAD. The cloning of DEDD and the prediction of an ICAD-like inhibitor for DEDD that is also cleaved by a caspase makes it likely that this system of translocating proteins to the nucleus is a common principle that is specific for apoptosis signaling.

Materials and methods

EST searches and cloning cDNAs encoding human and murine DEDD

To search for new DED-containing proteins, the program TFindPatterns in a modified version of GCG'5 FindPattern was used, which allowed a search for patterns in translated nucleic acid sequences databases (Senger *et al.*, 1995). For cloning of the human and murine cDNAs coding for DEDD, total RNA isolated from Jurkat J16 and murine EL4 cells were reverse-transcribed using the Perkin Elmer RT–PCR kit according to the manufacturer's protocol using PCR-primer 1 CGCGGATCCGGGGAAAGCATTGCGTACCTGAGCC, PCR-primer 2 CCGGAATTCC GGACAGTCCCCAAAGTGAGAAAGAGGG, PCR-primer 1-murine CGCGGATCCGGGC TCTATTTCTGAGCCTCTAGC and PCR-primer 2-murine CCGGAATTCCGGCAGAGGT GACGGAGCGAACAGTCC. For RT–PCR of human DEDD, primers 1 and 2 contained a *Bam*HI restriction and an *Eco*RI restriction site respectively at their 5′ ends. The amplified cDNA was cloned (*Bam*HI–*Eco*RI) into the multiple cloning site of pcDNA3. Sequence alignments and hydrophilicity blot were performed using the DNAStar sequence analysis software package, Lasergene. The nucleotide sequences of human and mouse DEDD have been submitted to the DDBJ/EMBL/GenBank database under the accession numbers AJ10973 and AJ011386, respectively.

Northern blot analysis and RT–PCR

Multiple poly(A)$^+$ RNA Tissue Northern (MTN^{TM}) Blot membrane (Clontech) was hybridized with a ^{32}P-labeled DNA probe of DEDD spanning the first 470 bp. Hybridization was performed according to the manufacturer's instructions. RT–PCR of total RNA isolated from different cell lines of lymphoid and non-lymphoid origin was performed using the Perkin Elmer RT–PCR kit according to the manufacturer's protocol. The RT–PCR probes were used in a competitive PCR (1 min×95°C, 1 min×59°C, 1 min×72°C, 35 cycles) using the primer 3 (CGCGGATCCGGGAGCATGGCGGGCCTAAAGCGGCG), primer 4 (CCGGAATTCCGG CTTGGTTCTGGATCACTGAAGGC) and β-actin primers.

Generation of DEDD mutant constructs

Using standard PCR and cloning techniques, the following constructs were generated: 3′FLAG-DEDD (primer 1 and primer 5 CCGGAATTCCGGTCACTTGTC ATCGTCGTCCTTGTAATCGGGCAAAGCTTCAGCATC), 5′FLAG-DEDD (primer 6 CGCGGATCCGCGATGGATTACAAGGACGACGATGACAAGATGGCGGGCCTAAAGCGGCG and primer 2), 3′FLAG-N-DEDD (amino acids 1–114) (primer 1 and primer 7 CCGGAATTCCGGTCACTTGTCATCGTCGTCCTTGTAATCTACAAGATCAGGGCACACAGC), and 5′FLAG-C-DEDD (amino acids 109–318) (primer 8 CGCGGATCCGCGATGG ATTACAAGGACGACGATGACAAGCGGGCTGTGTGCCCTGATC and primer 2). To generate GST fusion proteins, standard PCR and cloning techniques were applied. The GST–DEDD fusion protein was generated as described previously (Medema *et al.*, 1998). Generation of GST–DEDD, GST–FADD, GST–C-FADD (amino acids 89–207), GST–N-FADD (amino acids 1–88), GST–caspase-8, GST–N-caspase-8 (amino acids 1–180) and GST–C-caspase-8 (amino acids 181–478) was described recently (Medema *et al.*, 1998).

Transfection of 293T cells and cytotoxicity assay

Transfection was performed by using the calcium phosphate precipitation method. Transfected 293T cells were harvested after 36 h and DNA fragmentation as a specific measure of apoptosis was quantified as described previously (Peter *et al.*, 1995).

Generation of polyclonal anti-DEDD anti-serum

Polyclonal rabbit anti-DEDD antiserum was generated and affinity-purified as described previously (Peter *et al.*, 1995). The peptide CRAFSDPEPRPPQPSKTGPPH which corresponds to amino acids 139–158 of the EST clone Aa124451 (positions 129–148 of the DEDD primary sequence as found in DDBJ/EMBL/GenBank) was used for immunization. Resequencing of the clone and cloning of DEDD from Jurkat and EL4 cells showed two missequenced codons within this stretch of amino acids. Phe141 and Gly155 were corrected to Leu141 and Val155, respectively. These two conservative exchanges, however, did not affect the specificity of the anti-DEDD antibody.

Western blotting and peptide competition

Proteins were separated by 12% SDS–PAGE, transferred to Hybond nitrocellulose membranes (Amersham), blocked with 5% milk in phosphate-buffered saline with 0.05% Tween 20 (PBS/Tween) for 1 h, washed with PBS/Tween and incubated with the following antibodies: monoclonal anti-caspase-8 antibody C15 (IgG2b) (Scaffidi *et al.*, 1997), monoclonal anti-FADD antibody (IgG1) (Signal Transduction Lab., Lexington, KY), monoclonal anti-FADD antibody 1B5 (IgG2a) (unpublished data) and affinity-purified polyclonal anti-DEDD antiserum each used at a concentration of 1 μg/ml in 5% milk in PBS/Tween 20. The blots were washed with PBS/Tween and developed with goat anti-rabbit IgG (1:5000) or with isotype-specific goat anti-mouse antibodies (1:5000) in 5% milk PBS/Tween. After washing with PBS/Tween, the blots were developed with the Renaissance chemiluminescence system following the manufacturer's protocol (NEN, Boston, MA). For peptide competition, the polyclonal anti-DEDD antiserum was preincubated with 10 μM DEDD-peptide used for immunization (see generation of polyclonal anti-DEDD antiserum) for 30 min and then applied to the Western blot membrane like the non-peptide-treated antiserum. To quantify the amount

of DEDD in cellular lysates, the anti-DEDD antibody was used in a Western blot analysis to compare the signal obtained from endogenous DEDD with defined amounts of GST–DEDD.

Subcellular fractionation

Subcellular fractionation was performed as described previously (Martin and Green, 1996), though slightly modified. CEM cells (1×10^8) were stimulated with anti-APO-1 (1 μg/ml) and washed twice with PBS. Half of the cells were used for preparation of cytoplasmic and nuclear extracts, respectively. For preparation of cytoplasmic extracts, the cells were resuspended in 400 μl Cyt-buffer [50 mM HEPES pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed to swell by incubation for 20 min on ice. The cells were gently broken up using a Dounce homogenizer. Small aliquots of the lysate were taken and stained with trypan blue to determine the progression of cell lysis. Homogenization was continued until >95% of the cells were broken. After centrifugation at 14 000 *g*, the clear supernatant was carefully removed and supplemented with 100 μl of 5-fold concentrated standard reducing sample buffer (5× RSB). For preparation of nuclei, cells were incubated on ice for 20 min in Nuc-buffer (10 mM HEPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, 1 mM PMSF) and homogenized as described for the preparation of cytoplasmic extracts. When >95% of the cells were lysed, the salt concentration was adjusted to 150 mM, and the suspension was layered over 30% (w/v) sucrose in Nuc-buffer and centrifuged at 800 *g*. For preparation of nuclei from 293T cells, a 20% (w/v) sucrose solution was used. The nuclei were washed in Nuc-buffer, resuspended in 400 μl Nuc-buffer plus 100 μl of 5× RSB and 50 μl of each sample (nucleic and cytoplasmic extracts) were applied to 12% SDS–PAGE. In some experiments, cells were fractionated into microsolic, nuclear and cytoplasmic fractions as described elsewhere (Hsu *et al.*, 1997).

Immunofluorescence microscopy

293T cells transfected with FLAG-tagged DEDD-wild-type and DEDD-mutant were washed three times with PBS/1 mM $MgCl_2$ and fixed with methanol:acetone (1:1) at –20°C for 15 min. For co-staining experiments with anti-UBF antiserum, fixation was performed in 1.75% formaldehyde. The coverslips were allowed to dry, rehydrated with PBS/1 mM $MgCl_2$ and incubated with the monoclonal anti-FLAG antibody M2 (IgG1) (Sigma) or polyclonal human anti-UBF antibodies. After washing three times with PBS/1 mM $MgCl_2$ and incubation with the secondary antibody (goat anti-mouse IgG1-FITC for anti-FLAG and goat anti-human-Texas red for anti-UBF), the nuclei were stained with Hoechst 33258 (Sigma) (4 μg/ml in PBS/1 mM $MgCl_2$). After rinsing with PBS/1 mM $MgCl_2$, the coverslips were placed in 100% ethanol for a few seconds. After drying, the coverslips were mounted onto glass slides and photographs taken using standard fluorescence microscopy (Leitz DMRBE), or for co-staining experiments, confocal microscopy (LSM 310, Zeiss, Jena).

In vitro association assay of DEDD with caspase-8 and FADD

Using a TNT-coupled reticulocyte lysate system (Promega), DEDD, caspase-8 and FADD were *in vitro*-translated in the presence of [^{35}S]methionine. *In vitro*-translated proteins were incubated with 20 μg of GST fusion proteins precoupled to GSH beads for 2 h at 4°C in lysis buffer (20 mM Tris–HCl pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF and 1 mg/ml of leupeptin, antipain, chymostatin and pepstatin A). Subsequently, beads were washed three times with 20 volumes of lysis buffer and associated proteins analyzed by 12% SDS–PAGE followed by autoradiography.

In vivo association assay of DEDD with caspase-8 and FADD

Postnuclear supernatants of 10^8 untreated or anti-APO-1 treated Jurkat or 293T cells transfected with 3′FLAG-DEDD and crmA were prepared as described previously (Kischkel *et al*., 1995). The postnuclear supernatants were then added to 15 μg of the following mAbs: anti-caspase-8 C1 (Scaffidi *et al.*, 1997) or anti-FADD 1C4 (unpublished data) both coupled to anti-IgG1 agarose beads (Sigma) and incubated at least for 12 h at 4°C. Beads were then washed three times with Cyt-buffer. The immunoprecipitates were separated on 12% SDS–PAGE.

DNA labeling and nucleosome assembly

DNA was labeled for electromobility shift assays by PCRs containing [α-^{32}P]dCTP. The 248 bp fragment was amplified from a cloned rDNA fragment with primers spanning regions from –232 to –212 and –7 to +16 relative to the transcription start point, respectively.

Nucleosomes were assembled onto these fragments by transfer of histones bound to polyglutamic acid as described previously (Stein, 1989; Längst *et al.*, 1998).

Electromobility shift assay

1 μg GST–DEDD or GST–FADD were incubated for 10 min at room temperature with 1 μg DNA from phage λ in 40 μl of buffer (50 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl) containing different NaCl concentrations. Formation of DNA–protein complexes was analyzed on 1% agarose gels. To analyze binding to an oligonucleotide containing the murine rDNA promoter, a 248 bp fragment was amplified encompassing rDNA sequences from –232 to +16 relative to the transcription start site. 200–600 ng of the 5′-end-labeled fragment (300 000 c.p.m. per μg) were assembled into mononucleosomes by transfer of purified core histones complexed with polyglutamic acid (Stein, 1989; Längst *et al.*, 1998). 50 ng of free DNA or nucleosomal equivalents were incubated in a final volume of 20 μl with different amounts of GST–DEDD or GST–FADD in 50 mM Tris–HCl pH 8.3 for 10 min at room temperature. Then 5 μl of 20% glycerol were added and the DNA–protein complexes formed analyzed on 4.5% polyacrylamide gels in 0.5× TBE.

In vitro transcription

In vitro transcription on nucleosomal templates was performed essentially as described by Längst *et al.* (1997). The template used was $pMrT_2$, an artificial ribosomal minigene construct in which 5′-terminal murine rDNA sequences (from –168 to +155 relative to the transcription start site) were fused to a 3′-terminal fragment (from +603 to +686 with respect to the 3′ end of 28S RNA) containing the terminator element T_2 (Längst *et al.*, 1997). Chromatin was assembled onto 200 ng of $pMrT_2$ by incubation with 12 μl of an extract derived from early *Drosophila* embryos in a total volume of 40 μl for 5.5 h (Becker, 1994). The reaction was supplemented with 100 ng of recombinant His-tagged TTFΔ185 at the onset of assembly. GST–DEDD and GST–FADD were added to 25 μl transcription assays containing 12 mM Tris–HCl pH 7.9, 0.1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, CTP and GTP, 0.01 mM UTP, 1.5 μCi [α-^{32}P]UTP, 20 ng chromatin, 50 ng recombinant TTF-I and 4 μl of a murine protein fraction (DEAE-280 fraction) which contains all factors required for transcription initiation on rDNA templates. Reactions were incubated for 1 h at 30°C and stopped by adding one volume of 0.4 M ammonium acetate pH 5.5, 0.4% SDS and 0.2 mg/ml yeast tRNA. Transcripts were extracted and analyzed on 4.5% polyacrylamide gels.

Acknowledgements

We thank D.Süss and U.Matiba for excellent technical assistance. We are grateful to K.Glatting and M.Ebeling for help with the database searches. We wish to thank R.Voit for providing us with the anti-UBF antiserum, and V.Dixit and A.Strasser for the cDNAs for FADD and crmA, respectively. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie, the Tumor Center Heidelberg/Mannheim and the Deutsche Leukämieforschungshilfe.

References

Becker,P.B. (1994) The establishment of active promoters in chromatin. *BioEssays*, **16**, 541–547.

Boldin,M.P., Varfolomeev,E.E., Pancer,Z., Mett,I.L., Camonis,J.H. and Wallach,D. (1995) A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.*, **270**, 7795–7798.

Boldin,M.P., Goncharov,T.M., Goltsev,Y.V. and Wallach,D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell*, **85**, 803–815.

Casiano,C.A. and Tan,E.M. (1996) Antinuclear autoantibodies: probes for defining proteolytic events associated with apoptosis. *Mol. Biol. Rep.*, **43**, 211–216.

Casiano,C.A., Martin,S.J., Green,D.R. and Tan,E.M. (1996) Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)-mediated T cell apoptosis. *J. Exp. Med.*, **184**, 765–770.

Casiano,C.A., Ochs,R.L. and Tan,E.M. (1998) Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes. *Cell Death Differ.*, **5**, 183–190.

Chan,E.K., Imai,H., Hamel,J.C. and Tan,E.M. (1991) Human autoantibody to RNA polymerase I transcription factor hUBF. Molecular identity of nucleolus organizer region autoantigen NOR-90 and ribosomal RNA transcription upstream binding factor. *J. Exp. Med.*, **174**, 1239–1244.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505–512.

Chinnaiyan,A.M., Tepper,C.G., Seldin,M.F., O'Rourke,K., Kischkel,F.C., Hellbardt,S., Krammer,P.H., Peter,M.E. and Dixit,V.M. (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.*, **271**, 4961–4965.

Croston,G.E., Kerrigan,L.A., Lira,L.M., Marshak,D.R. and Kadonaga,J.T. (1991) Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription. *Science*, **251**, 643–649.

Eberstadt,M., Huang,B., Chen,Z., Meadows,R.P., Ng,S.C., Zheng,L., Lenardo,M.J. and Fesik,S.W. (1998) NMR structure and mutagenesis of the FADD (Mort1) death-effector domain. *Nature*, **392**, 941–945.

Enari,M., Sakahira,H., Yokoyama,H., Okawa,K., Iwamatsu,A. and Nagata,S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, **391**, 43–50.

Fernandes-Alnemri,T. *et al*. (1996) *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl Acad. Sci. USA*, **93**, 7464–7469.

Goltsev,Y.V., Kovalenko,A.V., Arnold,E., Varfolomeev,E.E., Brodianskii,V.M. and Wallach,D. (1997) CASH, a novel caspase homologue with death effector domains. *J. Biol. Chem.*, **272**, 19641–19644.

Han,D.K., Chaudhary,P.M., Wright,M.E., Friedman,C., Trask,B.J., Riedel,R.T., Baskin,D.G., Schwartz,S.M. and Hood,L. (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and Bcl-x_L and initiates cell death. *Proc. Natl Acad. Sci. USA*, **94**, 11333–11338.

Houge,G., Robaye,B., Eikhom,T.S., Golstein,J., Mellgren,G., Gjertsen,B.T., Lanotte,M. and Doskeland,S.O. (1995) Fine mapping of 28S rRNA sites specifically cleaved in cells undergoing apoptosis. *Mol. Cell. Biol.*, **4**, 2051–2062.

Hsu,Y.T., Wolter,K.G. and Youle,R.J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-x_L during apoptosis. *Proc. Natl Acad. Sci. USA*, **94**, 3668–3672.

Hu,S., Vincenz,C., Ni,J., Gentz,R. and Dixit,V.M. (1997) I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J. Biol. Chem.*, **272**, 17255–17257.

Inohara,N., Koseki,T., Hu,Y., Chen,S. and Nunez,G. (1997) CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc. Natl Acad. Sci. USA*, **94**, 10717–10722.

Irmler,M. *et al*. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature*, **388**, 190–195.

Kischkel,F.C., Hellbardt,S., Behrmann,I., Germer,M., Pawlita,M., Krammer,P.H. and Peter,M.E. (1995) Cytotoxicity-dependent APO-1(Fas/CD95)-associated proteins form a Death-Inducing Signalling Complex (DISC) with the Receptor. *EMBO J.*, **14**, 5579–5588.

Kuhn,A. and Grummt,I. (1992) Dual role of the nucleolar transcription factor UBF: trans-activator and antirepressor. *Proc. Natl Acad. Sci. USA*, **89**, 7340–7344.

Längst,G., Blank,T.A., Becker,P.B. and Grummt,I. (1997) RNA polymerase I transcription on nucleosomal templates: the transcription termination factor TTF-I induces chromatin remodeling and relieves transcriptional repression. *EMBO J.*, **16**, 760–768.

Längst,G., Becker, P.B. and Grummt,I. (1998) TTF-I determines the chromatin architecture of the active rDNA promoter. *EMBO J.*, **17**, 3135–3145.

Liu,X., Zou,H., Slaughter,C. and Wang,X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, **89**, 175–184.

Luger,K. and Richmond,T.J. (1998) DNA binding within the nucleosome core. *Curr. Opin. Struct. Biol.*, **8**, 33–40.

Martin,S.J. and Green,D.R. (1996) Cell-free apoptosis. In Cotter,T.G. and Martin,S.J. (eds), *Techniques in Apoptosis: A User's Guide*. Portland Press, London, pp. 121–130.

Medema,J.P., Scaffidi,C., Kischkel,F.C., Shevchenko,A., Mann,M., Krammer,P.H. and Peter, M.E. (1997) FLICE is activated by association with the CD95 Death-Inducing Signaling Complex (DISC). *EMBO J.*, **16**, 2794–2804.

Medema,J.P., Scaffidi,C., Krammer,P.H. and Peter,M.E. (1998) Bcl-x_L acts downstream of caspase-8 activation by the death-inducing signaling complex. *J. Biol. Chem.*, **273**, 3388–3393.

Muzio,M. *et al*. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex (DISC). *Cell*, **85**, 817–827.

Peter,M.E., Hellbardt,S., Schwartz-Albiez,R., Westendorp,M.O., Moldenhauer,G., Grell,M. and Krammer,P.H. (1995) Cell surface sialylation plays a role in modulating sensitivity towards APO-1-mediated apoptotic cell death. *Cell Death Differ.*, **2**, 163–171.

Peter,M.E, Medema,J.P. and Krammer,P.H. (1997) Does the *Caenorhabditis elegans* protein CED-4 contain a region of homology to the mammalian death effector domain? *Cell Death Differ.*, **4**, 523–525.

Peter,M.E., Scaffidi,C., Medema,J.P., Kischkel,F.C. and Krammer,P.H. (1998) The death receptors. In Kumar,S. (ed.), *Apoptosis, Problems and Diseases*. Springer, Heidelberg, pp. 25–63.

Rasper,D.M. *et al*. (1998) Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD95 (Fas, APO-1) receptor complex. *Cell Death Differ.*, **5**, 271–288.

Renz,A. and Fackelmayer, F.O. (1996) Purification and molecular cloning of the scaffold attachment factor B (SAF-B), a novel human nuclear protein that specifically binds to S/MAR-DNA. *Nucleic Acids Res.*, **24**, 843–849.

Rheaume,E., Cohen,L.Y., Uhlmann,F., Lazure,C., Alam,A., Hurwitz,J., Sekaly,R.P. and Denis,F. (1997) The large subunit of replication factor C is a substrate for caspase-3 *in vitro* and is cleaved by a caspase-3-like protease during Fas-mediated apoptosis. *EMBO J.*, **16**, 6346–6354.

Sakahira,H., Enari,M. and Nagata,S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, **391**, 96–99.

Scaffidi,C., Medema,J.P., Krammer,P.H. and Peter,M.E. (1997) Two FLICE isoforms, caspase 8/a and caspase 8/b, are recruited and activated by the CD95 Death-Inducing Signaling Complex (DISC). *J. Biol. Chem.*, **272**, 26953–26958.

Scaffidi,C. *et al*. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.*, **17**, 1675–1687.

Senger,M., Glatting,K.H., Ritter,O. and Suhai,S. (1995) X-HUSAR, an X-based graphical interface for the analysis of genomic sequences. *Comput. Methods Programs Biomed.*, **2**, 131–141.

Shaw,P.J. and Jordan,E.G. (1995) The nucleolus. *Annu. Rev. Cell Dev. Biol.*, **11**, 93–121.

Shields,J.M. and Yang,V.W. (1997) Two potent nuclear localization signals in the gut-enriched Krüppel-like factor define a subfamily of closely related Krüppel proteins. *J. Biol. Chem.*, **272**, 18504–18507.

Shu,H.B., Halpin,D.R. and Goeddel,D.V. (1997) Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity*, **6**, 751–763.

Smith,C.M. and Steitz,J.A. (1997) Sno storms in the nucleolus: new roles for myriad small RNPs. *Cell*, **89**, 669–672.

Song,Q., Lu,H., Zhang,N., Luckow,B., Shah,G., Poirier,G. and Lavin,M. (1997) Specific cleavage of the large subunit of replication factor C in apoptosis is mediated by CPP32-like protease. *Biochem. Biophys. Res. Commun.*, **233**, 343–348.

Srinivasula,S.M. *et al*. (1997) FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis. *J. Biol. Chem.*, **272**, 18542–18545.

Stein,A. (1989) Reconstitution of chromatin from purified components. *Methods Enzymol.*, **170**, 585–603.

Sudbeck,P. and Scherer,G. (1997) Two independent nuclear localization signals are present in the DNA-binding high-mobility group domains of SRY and SOX9. *J. Biol. Chem.*, **272**, 27848–27852.

Ubeda,M. and Habener,J.F. (1997) The large subunit of the DNA replication complex C (DSEB/RF-C140) cleaved and inactivated by caspase-3 (CPP32/YAMA) during Fas-induced apoptosis. *J. Biol. Chem.*, **272**, 19562–19568.

Vincenz,C. and Dixit,V.M. (1997) Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J. Biol. Chem.*, **272**, 6578–6583.

Ylikomi,T., Bocquel,M.T., Berry,M., Gronemeyer,H. and Chambon,P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.*, **11**, 3681–3694.

Zhou,B.B., Li,H., Yuan,J. and Kirschner,M.W. (1998) Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells. *Proc. Natl Acad. Sci. USA*, **95**, 6785–6790.

Received July 2, 1998; revised August 26, 1998; accepted August 27, 1998