Homophilic interactions of death effector domains (DEDs) are crucial for the signaling pathways of death receptor-mediated apoptosis. The machinery that regulates proper oligomerization and autoactivation of procaspase-8 and/or procaspase-10 during T lymphocyte activation determines whether the cells will undergo caspase-mediated apoptosis or proliferation. We screened a yeast two-hybrid library by using the DEDDs contained in the prodomains of procaspase-8 and procaspase-10 and isolated a DED-associated factor (DEDAF) that interacts with several DED-containing proteins but does not itself contain a DED. DEDAF is highly conserved between human and mouse (98% amino acid identity) and is homologous to a nuclear regulatory protein YAF-2. DEDAF is expressed at the highest levels in lymphoid tissues and placenta. DEDAF interacts with FADD, procaspase-8, and procaspase-10 in the cytosol as well as with the DED-containing DNA-binding protein (DEDD) in the nucleus. At the cell membrane, DEDAF augmented the formation of CD95-FADD-caspase-8 complexes and enhanced death receptor- as well as DED-mediated apoptosis. In the nucleus, DEDAF caused the DEDD protein to relocalize from subnuclear structures to a diffuse distribution in the nucleoplasm. Our data therefore suggest that DEDAF may be involved in the regulation of both cytoplasmic and nuclear events of apoptosis.

Apoptosis induction through death receptors can be triggered by formation of the death-inducing signaling complex (DISC) (1). CD95 (also known as FAS/APO-1) is a trimeric type I transmembrane receptor in the tumor necrosis factor receptor superfamily with a cytoplasmic tail that harbors a death domain (DD) that promotes DISC formation (2, 3). In the case of CD95-induced apoptosis, there are at least three components that form the DISC (1), including the CD95 cytoplasmic tail, an adapter molecule FADD/Mort-1, and caspase-8 and/or -10. The aggregation of the caspase-8 zymogen leads to autoactivation of this protease which triggers downstream apoptosis events (4, 5). FADD/Mort-1 is a cytoplasmic adapter protein that has a C-terminal DD that forms a homotypic interaction with CD95 and an N-terminal death effector domain (DED) that can homotypically associate with DED-containing caspases (6). Under appropriate conditions, FADD is recruited to the cytoplasmic tail of CD95 through a homotypic association between the DDs of CD95 and FADD. Procaspase-8 and procaspase-10 are two aspartate-requiring cysteine proteases that are each composed of two DED domains and a caspase domain that contains the large and small subunits of the protease. Again homophilic interactions between the DEDDs of FADD and procaspase-8/procaspase-10 allow these caspases to be recruited into DISC and undergo proteolytic autoactivation which causes apoptosis (7–9).

The DED has a characteristic structure composed of a bundle of six highly conserved α-helices (10). This tertiary structure is shared by two other homophilic interaction domains involved in apoptosis signaling, the “caspase recruitment domain” (CARD) and the “death domain” (DD), despite the low level of amino acid similarity between these domains (11). The CARD domain is found in the N-terminal domain of a subset of caspases as well as adapter proteins that are involved in the mitochondrial pathway of apoptosis. The DD is found in the cytoplasmic domains of death receptors of the tumor necrosis factor receptor superfamily as well as the various adapter proteins such as FADD, TRADD, and RIP that interact with these receptors. These domains are typically encoded by a single exon. Thus, these domains may have been derived from a common prototype domain that has become specialized in evolution for different cellular functions including apoptosis, activation, and inflammatory response (12). DEDDs have a pivotal role in apoptosis signaling interactions and can bind viral and cellular proteins that contain DEDDs that inhibit apoptosis. Therefore new molecules that interact with DEDD-containing proteins are good candidates for playing novel roles in regulating apoptosis and other cellular functions.

Recently, a DED-containing DNA-binding protein (DEDD) was identified (13). It has been shown that DEDD resides in cytoplasm but can be found to translocate into nucleus after CD95 stimulation and caspase activation. DEDD does not associate with the DISC; however, its nuclear translocalization is toxic to cells. DEDD localizes to the nucleolus and can associate with the RNA polymerase I transcription machinery. DEDD also has the ability to bind to DNA with no apparent sequence specificity. By protein sequence comparison, DEDD is likely to contain the same prototypical six α-helical domains as the DEDD of FADD, caspase-8, and -10. It is therefore interesting to examine whether other nuclear factors can regulate the function of DEDD.

To search for new molecules that potentially regulate death receptor-mediated apoptosis, we used the DEDD domains of procaspase-8 and procaspase-10 as molecular baits to screen hu-
man cDNA libraries by the yeast two-hybrid system. A novel protein, the DED-associated factor (DEDAF) that associates with DED-containing proteins but does not itself contain a DED was identified and analyzed.

The human DEDAF protein is composed of 228 amino acids and is almost identical to mouse RYBP (Ring-1- and YY-1-binding protein) which is a 228-amino acid basic protein (14). RYBP shares 75.6% similarity and 69.4% identity with another nuclear factor YAF2 (YY-1-associated factor 2) at the protein level. Expressed as a nuclear protein, YAF2 itself does not bind DNA, but it regulates the transcription of multiple genes through its association with the transcription factor YY-1 (Yin-Yang 1) and other components of the polycomb complex (14). Our observations suggest that DEDAF is a general regulator of DED proteins since it interacts with the DEDs of caspase-8 and -10 in the cytosol, as well as with DEDD in the nucleus. Over-expression of DEDAF in mammalian cells enhances CD95-mediated, as well as DED-mediated apoptosis.

MATERIALS AND METHODS

Yeast Two-hybrid Cloning—Human cDNA was made from a pool of mRNA derived from resting and 6-h phosphorylated 15-bromo-2-naphtol-1,7-dione-stimulated peripheral blood lymphocytes. The cDNA was linked into the HybriZAP vector and converted to a PAD-GAL4 library according to the manufacturer's instructions (Stratagene). The yeast strain YRG-2 was transformed with pBD-C8/C10.DED by the transformation method (Bio 101, Inc., Vista, CA) and selected with growth medium lacking tryptophan. The bait-transformed YRG-2 cells were subsequently transfected with the human T cell-derived PAD-GAL4 library and selected with tryptophan-, leucine-, and depleted histidine-medium for 5 days. Selected colonies were transferred onto a nitrocellulose membrane and subjected to a β-galactosidase assay according to Stratagene's instruction manual. Plasmids were retrieved from the positive clones by glass bead vortexing methods (15). The protein interactions were verified by switching the bait and prey constructs and then checking their “drop-out” resistance and induction of β-galactosidase activity. Inserts from the verified plasmids were sequenced and searched in the NCBI GenBank database (16).

Northern Blotting—Human multiple tissue blots were obtained from CLONTECH. Northern blotting assay was performed according to the manufacturer's manual using human DEDAF full-length cDNA as the probe that was labeled with 32P by Ready-To-Go™ beads following the manufacturer's protocols (Amersham Pharmacia Biotech).

Cells and Transfections—Human T cell lymphoma Jurkat-T and H9 cells were cultured in EHA (Biofluid, Rockville, MD) complete medium. A 20% fetal calf serum medium was used to culture cells the day before transfection. Stable DEDAF expressing lines were generated by transfecting Jurkat-T and H9 cells with pCMneo-DEDAF and selecting with G418 (Life Technologies, Inc.). HeLa, Chinese hamster ovary (CHO) cells, and human embryonic kidney kidney line 293T cells were cultured with Dulbecco's modified Eagle's complete medium and kept at 50–70% confluence for transfection.

Suspension cells were transfected by the electroporation method as described previously (5). The adherent cells were mostly transfected by using “Fugene 6” reagent according to the manufacturer's instructions (Roche Molecular Biochemicals).

Expression Plasmids and Antibodies—Mamalian expression plasmids were constructed onto pCMneo backbone (Promega) with pCMneo-DEDAF and selecting with G418. Unconjugated anti-HA monoclonal antibody was from Babco/Covance. Unconjugated anti-HA monoclonal antibody was from Roche Molecular Biochemicals and the anti-FLAG monoclonal antibody M2 (8 g/ml) was added into culture for 1 h, and the cells were washed twice with PBS, 1 mM MgCl2, and then stained with 1 μg/ml FITC-HA.111 epitope antibody in PBT buffer for 1 h at room temperature. The stained cells were fixed twice with PBT and briefly rinsed with PBT before mounting onto slides (Electron Microscopy Sciences) for fluorescence microscopy. For immunofluorescence analysis of DEDAF/DEDD interactions, 293T cells were cotransfected with HA-tagged DEDAF and 3'-FLAG-tagged DEDD by using the calcium precipitation method as described previously (13). The cells were grown on poly-prep slides (Sigma), transfected, washed three times with PBS, 1 mM MgCl2, and fixed with 3% paraformaldehyde at –20 °C for 4 min. The slides were allowed to dry, rehydrated with PBS, and incubated for 1–2 h at room temperature with anti-FLAG monoclonal antibody M2 (8 μg/ml). After washing three times with PBS, 1 mM MgCl2, the slides were incubated with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) (1:200) for 1 h at room temperature, again washed three times with PBS, 1 mM MgCl2, and placed in distilled water and 100% ethanol for a few seconds. After drying, coverslips were mounted onto the slides using Vectashield mounting medium (Vector Laboratories). The stainings were analyzed and documented using an Axiovert S100 immunofluorescence microscope (Zeiss) equipped with an AxioCam digital camera and software (Zeiss).

Results

Cloning and Characterization of DEDAF—To identify molecules that interact with DEDs and potentially regulate the activity of procaspases-8 and -10, we fused cDNA sequences of GAL4 DNA binding domain (BD) with the sequences coding for lysis buffer (30 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) with 1× protease inhibitor mixture (Roche Molecular Biochemicals). Lysates were immunoprecipitated for 2 h at 8 °C by appropriate antibodies and buffer-balanced protein G-Sepharose beads (Amersham Pharmacia Biotech). The immunoprecipitated beads were washed with TNTG buffer and subjected to 20% SDS-polyacrylamide gel electrophoresis under reducing conditions. For immunoprecipitation of nuclear proteins from cell extracts, the transfected 293T cells were resuspended in ice-cold lysis buffer (250 mM NaCl, 50 mM HEPES, pH 7.0, 5 mM EDTA, Complete™ protease inhibitors (Roche Molecular Biochemicals)) containing 0.1% Nonidet P-40 and tumbled for 1 h at 30 °C. The lysates were sonicated and cleared of debris by centrifuging at 4 °C with 14,000 × g. To immunoprecipitate DEDAF, the extracts were first precleared with 10 μg of normal mouse IgG (Santa Cruz Biotechnology) bound to protein A-Sepharose (Sigma) for 2 h at 4 °C and then tumbled with 10 μg of anti-HA monoclonal antibody bound to protein A-Sepharose for 16 h at 4 °C. The protein A-Sepharose was washed three times with lysis buffer and boiled in reducing sample buffer. Lysates of DEDAF/DEDAF double-transfected cells and their immunoprecipitates were separated by 12.5% SDS-polyacrylamide gel electrophoresis. The gel-separated proteins were transferred onto nitrocellulose membranes and probed at room temperature for 2 h with specific first antibodies as indicated and appropriate horseradish peroxidase-conjugated secondary antibodies, along with 5 rounds of a 5-min PBS-T wash. The membranes were developed by enhanced chemiluminescence using the SuperSignal chemiluminescent system (Pierce).

Immunofluorescence Microscopy—Immunofluorescence microscopy was described previously (17). Briefly, HeLa or CHO cells were cultured on 12-mm circular cover glass with appropriate media reaching 50–70% confluence. The cells were transfected with HA-tagged DEDAF derivative plasmids using Fugene 6 and cultured for 24 h. The nucleus dye Hoechst 33342 (1 μg/ml) was added into culture for 1 h, and the cells were fixed at –20 °C for 15 min with methanol. The cells were washed twice with PBT buffer (PBS with 0.1% bovine serum albumin and 0.01% Tween 20) and then stained with 1 μg/ml FITC-HA.111 epitope antibody in PBT buffer for 1 h at room temperature. The stained cells were fixed twice with PBT and briefly rinsed with PBT before mounting onto slides (Electron Microscopy Sciences) for fluorescence microscopy. For immunofluorescence analysis of DEDAF/DEDD interactions, 293T cells were cotransfected with HA-tagged DEDAF and 3’-FLAG-tagged DEDD by using the calcium precipitation method as described previously (13). The cells were grown on poly-prep slides (Sigma), transfected, washed three times with PBS, 1 mM MgCl2, and fixed with 3% paraformaldehyde at –20 °C for 4 min. The slides were allowed to dry, rehydrated with PBS, and incubated for 1–2 h at room temperature with anti-FLAG monoclonal antibody M2 (8 μg/ml). After washing three times with PBS, 1 mM MgCl2, the slides were incubated with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) (1:200) for 1 h at room temperature, again washed three times with PBS, 1 mM MgCl2, and placed in distilled water and 100% ethanol for a few seconds. After drying, coverslips were mounted onto the slides using Vectashield mounting medium (Vector Laboratories). The stainings were analyzed and documented using an Axiovert S100 immunofluorescence microscope (Zeiss) equipped with an AxioCam digital camera and software (Zeiss).

Apoptosis Assay—Jurkat and H9 lymphoma cells were transiently transfected with the indicated expression plasmids along with marker plasmid pSRO-H-2L,b by electroporation as described previously (5). Cells were cultured in 8 ml of EHA complete medium for 16–20 h. Live cells were obtained through Ficoll gradient centrifugation. For apoptosis assays, 2.5 × 106 cells were treated with the indicated doses of anti-CD95 (CH11) antibody for 6–10 h. The DEDAF stably transfected Jurkat and H9 lines were directly treated with anti-CD95 or TRAIL. The harvested cells were stained with annexin V-FITC, anti-H-Ld-PE (PharMingen), and propidium iodide (Sigma). The CD95-mediated and spontaneous apoptosis was measured by fluorescence-activated cell sorter analysis under constant time acquisition mode (30 s/sample). The propidium iodide-excluded H-2L-positive population was analyzed for annexin V-positive (apoptotic) and -negative (live) correspondingly (the % loss of transfected live cells is shown in Fig. 5).

RESULTS

Cloning and Characterization of DEDAF—To identify molecules that interact with DEDs and potentially regulate the activity of procaspases-8 and -10, we fused cDNA sequences of DEDAF Regulates DED-mediated Apoptosis

шифф отображения может содержать иерархии, списки, либо другие элементы структуры, но они не должны включать фрагменты текста, которые могут возникнуть в процессе обработки.
the DEDs of procaspase-8 and procaspase-10 (Fig. 1A). The fusion pBD-GAL4 constructs were used as the baits to screen a yeast two-hybrid library that derived from activated human lymphocytes. The library represents 5 × 10^6 independent clones. More than 2 million individual colonies were screened through six transformations. Positive clones were selected under Trp^− Leu^− His^− triple-deleted media and subjected to a β-galactosidase assay. The positive clones were verified through re-transformation using switched bait and prey constructs, re-selected by depletion medium, and re-analyzed by a β-galactosidase assay. Five groups of DED-interactive molecules were isolated as follows: DEDAF (2 hits from screening by the long isoform of caspase-10 DED), FADD (2 hits from caspase-8 DED screening), ubiquitin-conjugating enzyme 9 (UBC9, 39 hits from screening by the long isoforms of both caspase-8 and caspase-10 DED), and 2 other groups that are currently under investigation. One DEDAF clone spanned a 1.04-kb sequence that encoded an open reading frame for 228 amino acids. As shown in Fig. 1, BLAST analysis against the non-redundant data base at National Center of Biotechnology Information (NCBI) revealed that this DEDAF clone is a novel

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**FIG. 1. Caspase-10 and the DEDAF-interacting protein.** A, schematic of the caspase-10 protein. Boxes indicate the DED1, DED2, large (p20), and small (p10) subunits of the enzyme. The caret indicates a protein isoform with 33 additional amino acids (aa) generated by alternative splicing, and QACQG is the amino acid sequence of the enzyme active site. B, conceptual protein sequence of human DEDAF and its alignment with YAF2. The putative C2-C2 zinc finger motif is underlined. DEDAF has a predicted molecular mass of 25 kDa and its GenBank™ accession number is AF179286. C, DEDAF mRNA expression in human tissues. Northern blots of RNA from multiple human tissues were probed with radiolabeled human DEDAF DNA probes. Arrows point to the 4.5-kb-specific DEDAF band. The same membranes were re-probed with a radiolabeled ubiquitin probe.

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2 L.-X. Zheng and M. J. Lenardo, unpublished data.
FIG. 2. The DEDAF protein interacts with DED-containing proteins in 293T cells. A, the whole cell lysates from the cells cotransfected with either pAU1:Casp-10, pHA.DEDAF, or both (indicated by plus signs) were immunoprecipitated (IP) by AU1 and Western-blotted (W) with anti-HA antibodies (upper panel), and the expression of full-length AU1:Casp-10 in the corresponding lysates was shown in the lower panel by probing with AU1 antibody. The active site mutant version of caspase-10 was used so that death induction would not remove the cells expressing caspase-10. Vct indicates the expression vector not containing a DEDAF-coding insert. B, cotransfection of 293T cells with pHA.DEDAF and pAU1:Casp-8 or pAU1:FADD as indicated, and the cell lysates were subjected to immunoprecipitation with anti-HA(DEDAF) and Western blotting with anti-caspase-8 (C15) or anti-FADD, respectively. C, direct association of DEDAF with DEDD in nuclei of 293T cells. Cells were transfected with FLAG-DEDD (wt) + HA-DEDAF or FLAG-DEDD(F47Y) + HA-DEDAF. The antibodies used for immunoprecipitation and Western blotting (WB) are as shown. HC indicates immunoglobulin heavy chain.

full-length cDNA that shares 75.6% similarity and 69.4% identity with mouse YAF2 (18) at the protein level. Human DEDAF protein is also 98.7% identical to mouse RYBP, a mouse protein that interacts with YAF2 and RING protein (14). Therefore, DEDAF is likely to be the human homologue of this protein. The human DEDAF protein contains 228 residues by conceptual translation and has an estimated molecular mass of 25 kDa. There is a C2-C2 zinc finger motif at the N terminus (Fig. 1B).

We found a ubiquitous tissue distribution for DEDAF mRNA as detected by dot blot analysis using the Human RNA Master BlotTM from CLONTECH (data not shown). Northern blot analysis of multiple human tissues revealed a 4.5-kb transcript with abundant expression in placenta and lymphoid tissues, suggesting a predominant expression in hematopoietic cells (Fig. 1C). Sequence contig analysis against the nucleotide data base from NCBI assembled a 4.5-kb sequence that correlates with the observed size in Northern blots. A 4.5-kb DNA was also obtained by nested reverse transcriptase-polymerase chain reaction, which confirms the result from Northern blot (data not shown). By using BLAST search against the newly available Human Genomic Draft data base, DEDAF was mapped to the short arm of chromosome 3 (3p12–13). The locus sequence revealed two small 5′-exons followed by a giant intron (67 kb) and three additional protein-coding exons. The DEDAF transcript has a long 3′-untranslated region.

**DEDAF Interacts with DED-containing Proteins.**—To confirm the interactions between caspase-10 DEDs and DEDAF in mammalian cells, we cotransfected 293T cells with AU1 epitope-tagged procaspase-10, AU epitope-tagged DEDs of caspase-10, and HA epitope-tagged DEDAF and then performed immunoprecipitation/Western blotting analysis. This transfection showed that, indeed, the DEDAF cDNA encoded a 25-kDa protein (correcting for the epitope tag) that coprecipitated with caspase-10 (Fig. 2A, lane 2, top panel). We also observed that DEDAF interacts with FADD and caspase-8 through their DEDs (Fig. 2B and data not shown). Moreover, DEDAF specifically associated with the DEDs of caspase-10 (see below). Since a significant portion of DEDAF is expressed within the nucleus, we tested whether DEDAF also bound DEDD, a DED protein that was recently shown also to be mainly expressed in the nucleus under these conditions (13). DEDAF also associates with DEDD (Fig. 2C). A point mutation within DEDD (F47Y) resulted in strongly diminished binding to DEDAF (Fig. 2C). Phenylalanine 47 lies within the N-terminal DED of DEDD. It has been shown previously that the replacement of the analogous conserved phenylalanine in the DED of FADD (P25Y) resulted in a reduced association between FADD and caspase-8 (10). In all experiments we carefully tested whether each coexpressed protein would affect the expression levels, and conditions were chosen that ensured similar expression. The data support the notion that DEDAF could regulate DED-containing proteins.

To map the DED interaction region of DEDAF, we tested a series of N- and C-terminal truncation mutants of DEDAF (see Fig. 3A) for their ability to associate with DEDs of caspase-10 in 293T cells. We found that the DEDAF association with caspase-10 DEDs was lost in the DEDAF-(1–144) deletion mutant and greatly reduced in the DEDAF-(45–227) and DEDAF-(67–227) truncations, indicating that the DED interaction site is likely in the central portion of the DEDAF molecule (Fig. 3B).

**Cellular Localization of DEDAF**—To study the cellular expression pattern of DEDAF, the N-terminal HA-tagged proteins of DEDAF and its mutants were expressed in 293T and CHO cells that were then examined by fluorescence microscopy. Similar results were obtained in both cell lines. A typical DEDAF expression pattern is shown in Fig. 4A. By using the Hoechst 33342 nuclear staining dye that defines nuclei, we observed that the majority of wild-type DEDAF protein is expressed in the nucleus as spread dots, although there is also evidence of DEDAF in the cytoplasm. However, the C-terminal truncated mutants, DEDAF-(1–112) and -(1–144), adopted a diffused and non-nuclear pattern as shown in the lower panels of Fig. 4A (for DEDAF-(1–144)). These data indicate that the C terminus of DEDAF is essential for nuclear localization.

We then transfected 293T cells with pDEDAF.wt:GFP and
pCasp.10-DED-RFP and examined their fluorescent protein expression (Fig. 4B). The result shows that caspase-10 DED is expressed in a unique perinuclear filamentous structure that was previously reported for the caspase-8 DEDs (17). We also noticed that the nuclear dotted DEDAF expression was dramatically altered when caspase-10 DEDs were coexpressed, in a manner indicating that the cells were undergoing apoptosis. There appears to be partial colocalization of the two molecules at the perinuclear region as shown by bright yellow overlap regions in the aligned picture (lower right panel of Fig. 4B). It was obvious however, that the overexpressed DEDAF and caspase-10 DEDs do not completely colocalize in the cells.

**DEDAF Affects the Subnuclear Distribution of DEDD—**

DEDD is not toxic until it is translocated into the nucleus and localizes to nucleoli-like structures (13). To examine the potential of DEDAF to regulate DEDD, we cotransfected DEDD with either wild-type or mutant DEDAF and then examined the expression pattern of DEDD. We found that coexpression of DEDD with wild-type DEDAF changed the localization of DEDD within the nucleus. In the presence of DEDAF, DEDD was found in the nucleoplasm and not in the nucleoli-like structures (Fig. 4C). This effect was even more pronounced when DEDAF was coexpressed with N-DEDD (a truncated DEDD mutant containing only the DED flanked by two of the three nuclear localization signals found in DEDD (13) (data not shown)), suggesting that DEDAF interacts with the DED in DEDD, which is consistent with the coimmunoprecipitation results of DEDD and its DED mutant F47Y as shown in Fig. 2B. Therefore, the data suggest that DEDAF might be an interaction partner for nuclear DEDD and support the notion that DEDAF is a protein that has the ability generally to bind to DED proteins both in the cytoplasm and in the nucleus. Moreover, the expression of DEDAF may control the subnuclear localization of DEDD and may thereby regulate the activity of DEDD to inhibit RNA polymerase I-dependent transcription that is controlled by DEDD.3

**DEDAF Regulates DED- and Death Receptor-mediated Cell Death—**

Cross-linking CD95 does not trigger formation of the DISC in 293T cells that express barely detectable endogenous CD95, FADD, and caspase-8. However, we observed a greatly augmented DISC formation when DEDAF and procaspase-8 were coexpressed (Fig. 5A). We also noticed that DEDAF could substantially increase the interactions between FADD and procaspase-8 (data not shown). These results suggest that DEDAF may be involved in regulation of DISC formation through enhancing FADD/caspases-8 or -10 association.

To test the role of DEDAF in death receptor-mediated apoptosis, Jurkat and H9 cells were transiently transfected with

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3 O. Schickling and M. E. Peter, unpublished data.
pHA.DEDAF, a cytomegalovirus promoter-driven DEDAF expression plasmid. We found that overexpression of DEDAF consistently rendered these T lymphoma cells more susceptible to anti-CD95-induced apoptosis. Similar phenomena were observed when examining the apoptosis response of Jurkat and H9 cells stably transfected with DEDAF to anti-CD95 stimulation (Fig. 5, B and C). However, overexpression of DEDAF-(1–144), a C-terminal truncation mutant that lost its nuclear
expression pattern and its association with the DED of caspase-10 (Fig. 3B), does not seem to interfere with anti-CD95-induced death in Jurkat cells (data not shown). We also found that apoptosis stimulated by TRAIL, another member of the tumor necrosis factor gene superfamily, was similarly enhanced (data not shown). Furthermore, we observed that the expression of DEDAF antisense RNA could substantially (20%) inhibit anti-CD95-induced death in H9 cells (Fig. 5D).

We have previously reported that overexpression of isolated caspase-8 DEDs could cause the formation of the death effector filaments that trigger HeLa cell apoptosis (17). To test the role of DEDAF on the DED-induced apoptosis, we cotransfected HeLa cells with DEDAF and caspase-10 DEDs expressing plasmids. Our data suggest that DEDAF overexpression alone has little effect on the viability of the transfected cells; however, a substantial dose-dependent enhancement of DED-mediated apoptosis was observed when caspase-10 DEDs and DEDAF were coexpressed (Fig. 5E and F).

**DISCUSSION**

The “death effector domain” (DED) is one form of the prototypical protein interaction domain that governs apoptosis pathways, and DED-containing molecules play important roles in death receptor-mediated apoptosis (19). FADD as an adapter protein contains one DED and serves to oligomerize procaspase-8, procaspase-10, and FLIP during death ligand occupancy of their receptors by recruiting these molecules to the DISC complex (6, 20). Procaspase-8, procaspase-10, and cFLIP all have two DED domains and are involved in regulating the auto-cleavage and activation of a caspase cascade that leads to apoptosis (7, 8, 21, 22). There are virus-encoded DED proteins that are capable of blocking caspase activation (23). All these facts point to the idea that oligomerization of the DED-containing caspases is required for an irreversible execution of death signals. However, it remains unclear how this process is regulated. We have identified DEDAF as a protein that interacts directly with the DEDs of FADD, procaspase-8, procaspase-10, and DEDD through a non-homotypic mechanism. By using 293T cells that have a very low level of endogenous CD95 and FADD expression, we found that DEDAF promotes CD95/ FADD/caspase-8 association (Fig. 5A and data not shown). This correlates with its ability to enhance CD95-induced DISC formation. DEDAF augments CD95- and TRAIL receptor-mediated apoptosis in a dose-dependent pattern (Fig. 5B and C and data not shown). Furthermore, we also observed that DEDAF antisense expression substantially protects H9 cells from CD95-induced apoptosis. Thus, DEDAF is potentially a positive regulator of DISC formation and enhances death signals mediated by death receptors at the cell membrane.

Recent studies suggest that the DEDs may have functions besides mediating classical CD95-induced apoptosis (13, 17, 24). We have previously reported that overexpression of DED-containing molecules could cause formation of the perinuclear death filaments that correlate well with apoptosis induction (17). Our recent data show that expression of the DED domains of caspase-10 could potently kill caspase-8-deficient Jurkat lymphoma cells through CD95 pathway, and this DED-medi-
ated cell death is hardly inhibitable by the pan caspase inhibitor benzoyloxycarbonyl-VAD suggesting it may not involve caspases.2 Furthermore, mammalian DED proteins could cause bacterial cell death by increasing reactive oxygen species (24). All these results indicate that DED proteins may participate in a distinct apoptosis pathway that needs to be clarified. We observed that coexpression of DEDAF and the prodomain of caspase-10 enhanced DED-mediated cell death (Fig. 5, E and F), indicating that DEDAF may also be involved in this alternative pathway.

DEDAF is a nuclear protein with an inhomogeneous dotted expression pattern as detected by fluorescent microscopy of the DEDAF:GFP fusion protein in transfected 293T, HeLa, and Jurkat cells (Fig. 4 and data not shown). Further truncation analysis indicates that this nuclear pattern correlates with its ability to associate the DED domains of procaspase-8 and procaspase-10. Mutants that lose the nuclear expression pattern also lose their association with caspase-10 DED (Fig. 3B and Fig. 4A). Moreover, DEDAF does not contain any obvious nuclear localization signals. However, when transiently overexpressed, it significantly localizes to the nucleus. In stable transfectants we found DEDAF expressed in the cytosol and the nucleus pointing to a function in both compartments (data not shown). DEDAF could therefore interact with both cytoplasmic and nuclear DED proteins.

DED has been found to be expressed in the nucleus and may regulate RNA polymerase I transcription as well as nuclear apoptosis (13). We have observed that DEDAF mutants lacking the typical nuclear expression pattern lose their abilities to retain DEDD in nucleoplasm (Fig. 4C). Therefore, DEDAF could regulate DEDD function by its interaction with the DED. Our data suggest that DEDAF associates with nuclear and cytoplasmic DED-containing molecules, promotes CD95-mediated DISC formation, and augments death receptors as well as DED-induced apoptosis. The direct interactions between DEDAF and these DED-containing molecules suggest that DEDAF regulates distinct events in the nucleus and cytoplasm.

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