The EMBO Journal Vol.18 No.2 pp.353–362, 1999

Structure–function analysis of an evolutionary conserved protein, DAP3, which mediates TNF-α- and Fas-induced cell death

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A novel approach to the isolation of positive mediators of programmed cell death, based on random inactivation of genes by expression of anti sense RNAs, was employed to identify mediators of interferon-γ-induced apoptosis. One of the several genes identified is *DAP3*, which codes for a 46 kDa protein with a potential nucleotide-binding motif. Structure–function studies of the protein indicate that the intact full-length protein is required for its ability to induce apoptosis when overexpressed. The N-terminal 230 amino acids, on the other hand, act in a dominant-negative fashion. Both of these functions are dependent on the integrity of the nucleotide binding motif. Expression of anti-sense DAP3 RNA and of the dominant interfering form of DAP3 both protected cells from apoptosis induced by activation of Fas and tumor necrosis factor α (TNF-α) receptors. Thus, DAP3 is implicated as a positive mediator of these death-inducing stimuli. It functions downstream of the receptor signaling complex and its death promoting effects depend on caspase activity. In the nematode *Caenorhabditis elegans*, a potential homolog of DAP3 showing 35% identity and 64% similarity to the human protein was isolated. Overexpression of the nematode DAP3 cDNA in mammalian cells induced cell death, indicating that the protein is conserved at the functional level as well as the structural level.

Keywords: anti-sense/apoptosis/DAP3/Fas/TNF-γ

Introduction

The process of apoptosis (programmed cell death) is genetically controlled and fundamental to the development and maintenance of all multicellular organisms. Aberrations of this process result in various pathologies including autoimmune diseases, neurodegenerative diseases and cancer. Various approaches have been employed in an attempt to identify genes that mediate apoptosis. A few research directions were based on the finding that the core mechanism of the apoptotic process is well conserved from worms to mammals. The identification of genes in the cell-death pathway through the study of mutants of the nematode *Caenorhabditis elegans* has led eventually to the identification of the homologous genes in mammalian cell-death pathways. Three of these nematode genes have been studied extensively: *ced-3*, *ced-4* and *ced-9* (reviewed in Hengartner and Horvitz, 1994). Their mammalian homologs were identified as the caspases (cysteine proteases), Apaf-1 and members of the Bcl-2 family, respectively. The fact that these protein families constitute the core of the apoptotic machinery in mammalian cell-death systems was subsequently established (reviewed in Peter *et al*., 1997). Another approach consisted of the isolation of proteins which are recruited to the intracellular domains of cytokine receptors belonging to the tumor necrosis factor (TNF) family. The yeast two-hybrid system served as a major tool for the isolation of these cytoplasmic proteins that bind to the intracellular part of TNF-α receptor (p55 TNF-R1), the Fas receptor, and to each other (Yuan, 1997; Ashkenazi and Dixit, 1998; Wallach *et al*., 1998). Other strategies employed were based on functional gene screening for positive mediators of cell death. These approaches utilized cDNA expression libraries coding for anti-sense RNA and/or dominant negative protein fragments to identify cell-death genes (Deiss and Kimchi, 1991; Gabig *et al*., 1994; Gudkov *et al*., 1994; Vito *et al*., 1996).

The functional gene screening employed in our laboratory [named Technical Knock Out (TKO)] is based on transfections of HeLa cells with an anti-sense cDNA library and exposure of the cell cultures to the apoptotic stimulus of interferon-γ (IFN-γ) (Deiss and Kimchi, 1991). Cells in which an anti-sense RNA reduced the expression of a positive mediator of apoptosis survived for longer time periods, while the rest died efficiently. This functional screen resulted in the isolation of several genes that were further characterized (reviewed in Kimchi, 1998; Kissil and Kimchi, 1998). Among these genes, we have identified a novel calcium/calmodulin-dependent serine/theronine kinase (DAP-kinase), which carries ankyrin repeats and a typical death domain (Deiss *et al*., 1995; Cohen *et al*., 1997) and DAP5, a novel homolog of translation initiation factor 4G (eIF4G; p220) (Levy-Strumpf *et al*., 1997). Another gene, *DAP3*, codes for a protein of 46 kDa which carries a potential 'P-loop' motif, suggesting that it is a nucleotide binding protein. The gene, which is localized to chromosome 1q21 (Kissil and Kimchi, 1997), is transcribed into a single 1.7 kb mRNA that is ubiquitously expressed in different tissues (Kissil *et al*., 1995). While the expression of DAP3 anti-sense RNA protected cells from death by IFN-γ, the overexpression of the full-length sense cDNA was alone sufficient to induce cell death in HeLa cells (Kissil *et al*., 1995). Here we report on the structure–function analysis of DAP3 protein. We provide a few independent lines of evidence showing that DAP3 is involved in TNF-α- and Fas-induced cell death, thus indicating that the function of this gene in cell death is wider than initially thought and is not exclusive to IFN-γ, the stimulus used in the initial selection. The functional

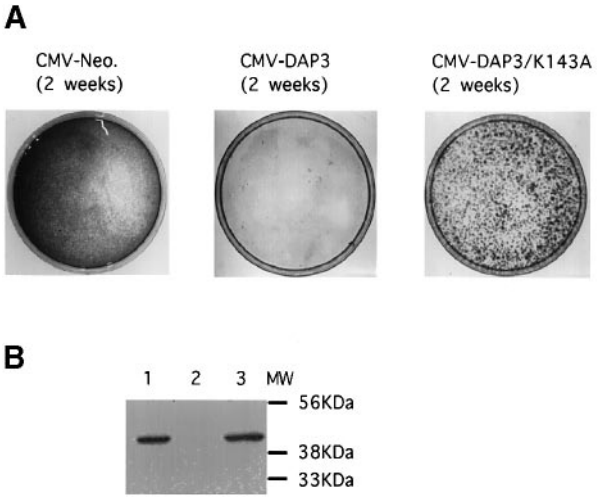

Fig. 1. The integrity of the P-loop is essential for DAP3 to induce its full death effect when overexpressed. (**A**) HeLa cells transfected with either wild-type or mutant DAP3 or with an empty control vector. Stably transfected cells were selected for with G418 for 2 weeks and numbers of colonies remaining on plates were scored after staining with crystal violet. Plates represent similar results from three experiments, performed in triplicate, with different plasmid preparations. (**B**) Expression of CMV–DAP3 and CMV–DAP3 mutant in 293 cells transiently transfected with the different constructs. Western blots were probed with anti FLAG-epitope antibodies. Lane 1, CMV–DAP3; lane 2, empty vector; lane 3, CMV–DAP3 mutant.

mapping indicates that DAP3 acts downstream of the receptor signaling complex and upstream to some caspases. In addition, we report on the identification of a homologous gene to *DAP3* from *C.elegans* and its functional similarity to the mammalian DAP3 when overexpressed in human cells.

Results

The P-loop mutant of DAP3 is defective in inducing cell death when overexpressed

In order to evaluate whether the nucleotide binding motif (P-loop) of DAP3 is essential for inducing cell death when DAP3 is overexpressed, a mutant of the DAP3 protein was generated. A point mutation was introduced into DAP3 cDNA causing a change in the amino acid sequence from lysine 143 to alanine within the P-loop. This type of mutation has been shown to abolish nucleotide binding abilities of various nucleotide binding proteins (reviewed in Saraste *et al*., 1990). Mutant and wild-type DAP3 were subcloned into pCDNA3 expression vector (containing resistance to G418) and transfected into HeLa cells. The transfected cells were selected in the presence of G418. After 2 weeks of selection, the plates were stained and numbers of colonies scored. As can be seen in Figure 1A, the overexpression of wild-type DAP3 was not compatible with colony growth and there was a massive reduction in number of colonies on this plate, as compared with cells that were transfected with an empty vector (see Table I). Examination of HeLa cells during the first days following transfection, by co-transfections with β-galactosidase (β-gal) to visualize the transfectants, fixation of the cultures and performance of enzymatic assays, revealed that ~35% of the transfectants had an apoptotic morphology at any given time point (data not shown). This suggested further that the reduction in colony number resulted from cell death.

Table I. DAP3 induces cell death when overexpressed in HeLa and 293T cells

Cell lines	Transfected vector	No. G418 resistant colonies/plate[a]
HeLa	DAP3–CMV	850
HeLa	DAP3mut–CMV	1×10^4
HeLa	control	3.8×10^5
		Percentage of apoptotic transfectants[b]
293	DAP3–CMV	35
293	DAP3mut–CMV	14
293	control	7.2

[a]Numbers of resistant HeLa colonies present on 9-cm plates, counted at day 10 after G418 (800 μg/ml) addition.
[b]Scoring under fluorescence microscopy as in Figure 2, 24 h post transfection. Only transfected cells were counted. Results represent the average of three separate transfections performed in triplicate.

Mutant DAP3, however, had significantly milder effects in comparison with wild-type DAP3. Although the number of colonies was still lower than the outcome of transfections with the control vector, it was substantially higher than the one obtained by transfections with wild-type DAP3 (Figure 1A). Thus, although not totally abolished, the death-inducing activity of DAP3 is severely impaired in the P-loop mutant form of the protein. Both forms of the DAP3 protein were expressed at similar levels in cells, suggesting that they do not differ in their translatability or stability (Figure 1B). Thus, the integrity of DAP3 nucleotide-binding motif is essential for DAP3 to exert its full inhibitory effects in the colony formation assay.

A method to quantify cell death in a simpler, faster and more direct assessment was then established. This assay was designed to observe, on a single cell basis, the events that occur after transfection, and the time course of cell death. In this type of assay, 293 human embryonic kidney (HEK) cells were transiently co-transfected with a vector expressing green fluorescent protein (GFP), and either a control empty vector or vectors driving the expression of wild-type DAP3 or the P-loop mutant. The GFP was used as a marker to visualize the transfected cells and to assess the apoptotic frequency among the transfectants according to morphological alterations. Approximately 24 h post transfection the morphology of the transfected cells was directly assessed by fluorescence microscopy. Apoptotic cells were scored by well defined alterations including membrane blebbing, rounding up and shrinkage of cells, and fragmentation into 'apoptotic bodies'. In addition, some of the transfected cells detached from the plate. As can be seen in Figure 2A and is summarized in Table I, expression of wild-type DAP3 in 293 cells induced apoptosis in 35% of the transfected cells 24 h after transfection. The non-specific background of cell death following transfections with an empty vector was ~7%. The DAP3 P-loop mutant was defective in this assay in its ability to induce cell death to a full extent. The transfection resulted in 14% apoptotic cells, scored according to the same morphological alterations. The assay was repeated several times with different plasmid preparations,

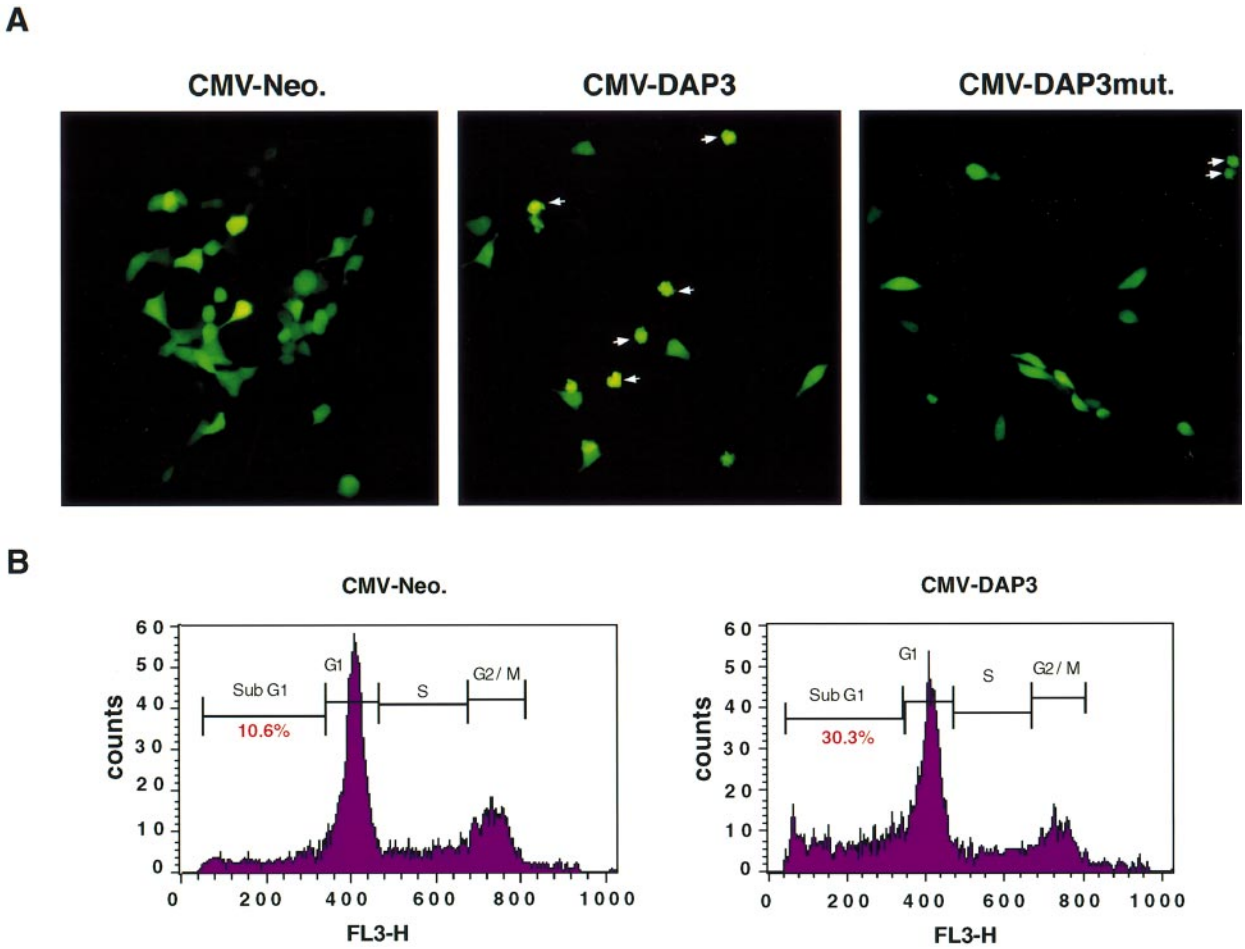

Fig. 2. Overexpression of wild-type DAP3 induces apoptosis in 293 HEK cells. (**A**) 293 HEK cells were transfected with a GFP expression vector and either CMV–DAP3, CMV–DAP3 mutant or an empty control vector (CMV–Neo.). Arrows indicate apoptotic cells. (**B**) DNA content distribution of REFs transfected with farnesylated GFP and either CMV–DAP3 or an empty control vector (CMV–Neo.). Histograms represent cell counts versus DNA content (FL3-H). The different cell-cycle phases and fraction of cells containing a sub-G_1 DNA content are indicated.

and the difference between the death-promoting effects of the two DAP3 forms was statistically significant, as well as the difference between the mutant DAP3 and non-specific background. Thus, while a functional P-loop contributes to the full capacity of DAP3 to promote cell death by overexpression, additional critical domains reside in the DAP3 protein.

To test whether normal primary cells are also sensitive to the death-inducing function of DAP3, as immortalized cell lines are, rat embryo fibroblasts (REFs) were transfected with CMV–DAP3 and 48 h later were stained with propidium iodide (PI) and analyzed by FACS for their DNA content. In the control transfections, an empty vector was used instead. The REFs were co-transfected with a vector expressing a membrane-localized form of GFP. Apoptosis was assessed by determining the number of cells within the GFP positive gated population that have a sub-G_1 DNA content, indicative of chromatin fragmentation. DAP3 transfection increased the number of REFs displaying a sub-G_1 DNA content from a background of 10.6 to 30.3% apoptotic cells (Figure 2B). The cell cycle distribution of the viable cells did not change. Thus, as in the HeLa and 293 cell lines, the expression of DAP3 in primary fibroblasts also induced an increase in apoptotic cells.

DAP3 anti-sense RNA protects HeLa cells from Fas-induced apoptosis

As DAP3 was isolated as a positive mediator of IFN-γ-induced cell death, it was interesting to see whether it might mediate apoptosis induced by other known external stimuli. To test this possibility, we first used the previously described HeLa cell polyclonal populations that are stably transfected with the Epstein–Barr virus (EBV)-based expression vector carrying DAP3 anti-sense cDNA fragment (Kissil *et al*., 1995). Two polyclonal populations expressing DAP3 anti-sense RNA, and a single control population expressing a non relevant gene, *DHFR*, were treated with anti-Fas agonistic antibodies, which have been shown to mimic the effect of Fas ligand and to effectively induce cell death (Trauth *et al*., 1989). The viability of the treated cell populations was assessed by a neutral red dye uptake assay. In the DHFR-transfected cell population the agonistic antibodies triggered cell death highly efficiently. By 28 h post addition of the anti-Fas antibodies to the growth medium, cell viability corresponded to 28% of the value measured in non-treated population. In contrast, the two cell populations expressing the DAP3 anti-sense RNA were significantly less sensitive to the killing effects of the antibodies and exhibited 55–60% viability at this time point (Figure 3). This

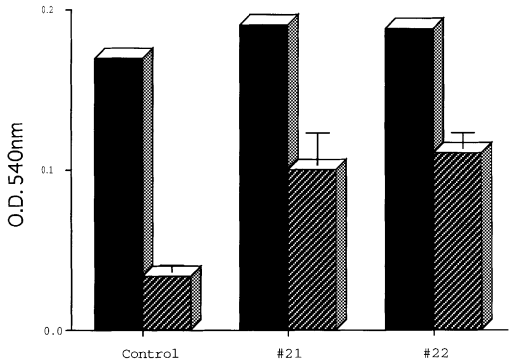

Fig. 3. DAP3 antisense RNA protects HeLa cells from Fas-induced cell death. Two polyclonal populations of HeLa cells that were stably transfected with a vector expressing the DAP3 antisense RNA (#21, #22) and a control polyclonal population of HeLa cells transfected with a vector expressing the DHFR RNA were used (control). Viability was scored by uptake of 'neutral red' dye, 28 h post treatment with the anti-Fas/APO-1 agonistic antibody. Results represent an average of three independent experiments performed in quadruplets. Untreated cells, solid black bars; treated cells, striped bars.

consistent pattern of cell death protection by DAP3 anti-sense RNA provided the first support for DAP3 being involved in Fas-induced apoptosis.

The 230 N-terminal amino acids of DAP3 act in a negative-dominant fashion

As the P-loop binding motif is the only recognizable motif that can be detected by computer programs such as MOTIF (GCG, Wisconsin package), it became clear that further analysis should depend on experimental dissections of the protein. Thus, to test for function of various regions of the DAP3 protein, two sets of N- and C-terminal deletion mutants were constructed. One set was prepared from the wild-type DAP3 cDNA and another from the P-loop mutant form of DAP3 cDNA. The various deletion mutants are presented in Figure 4A. All the mutants were first tested for expression in transiently transfected 293 cells. It is shown in Figure 4B that the various deletion mutants could be easily detected with anti-DAP-3 monoclonal antibodies (the antibodies are directed against a region which is common to all DAP3 fragments; the ratio between the full-length endogenous DAP3 and the ectopically expressed DAP3 fragments does not reflect the real ratio on a single-cell basis since only a small fraction of the analyzed population expresses the transgene). The deletion mutants were then assessed for either induction of apoptosis by mere ectopic expression or for suppression of apoptosis induced by external cytokines. The assays were done by co-transfection of the relevant vector(s) with a GFP expression vector and apoptosis was scored based on morphology as described above.

The ectopic expression by itself of all the deletion mutants had no significant effects on cell viability, and the percentage of apoptotic cells among the GFP positive cells did not differ significantly from that of background levels (summarized in Figure 4A). It is concluded from this study that truncation of a minimal region comprising 68 amino acids from the C-terminus, or 100 amino acids from the N-terminus (which does not reach the P-loop motif) was by itself sufficient to abolish completely the death-promoting function of wild-type DAP3 and to eliminate the residual activity that was retained by the P-loop mutant (Figure 4A). Thus, the death-promoting functions of DAP3 depend not only on the ability to bind a putative nucleotide, but also on the correct protein folding or the presence of other functional domains in the protein, each of which may be disrupted by the deletion mutations.

To score for possible death-protective effects of the deletion mutants (which may indicate that a certain fragment possesses dominant-negative features) they were each co-transfected into 293 cells with an expression vector encoding a chimeric receptor composed from the intracellular domain of the Fas receptor and the extracellular domain of the p55-TNF-R1 (p55/Fas). This chimera is a potent inducer of cell death via Fas signaling pathways (Boldin *et al*., 1997). The self aggregation of these receptors triggers Fas signaling without a need for a ligand or agonistic antibodies and the choice of this system was prompted by the results in Figure 3 which implicated DAP3 in Fas-induced cell death. When transfected alone, the p55/Fas induced apoptosis in 85% of transfected 293 cells at 24 h post transfection (Figure 5A). As a positive control for protection from Fas-induced apoptosis, a vector expressing the dominant negative form of the MORT-1/FADD adapter protein (named DN-MORT-1) was used. This protein binds directly to the intracellular domain of the Fas receptor and thus blocks Fas signaling at the receptor proximal level (Boldin *et al*., 1995; Chinnalyan *et al*., 1995). When introduced into cells together with p55/Fas the number of apoptotic cells among the transfectants dropped to 27% at 24 h post transfection, indicating that these transient assays can be used for scoring death-protective effects of various cDNA fragments.

Each of the DAP3 deletion mutants was then co-transfected with the p55/Fas chimera. All the tested N-terminal deletions failed to protect from Fas-induced apoptosis, as well as the smallest C-terminal deletion (Δ330) (Figure 4A). In contrast, Δ230 mutant (a mutant that lacks 168 C-terminal amino acids) was effective in death protection. It reduced the percentage of apoptotic cells from 85 to ~45% at 24 h post transfection (Figure 5A), highly similar to the extent of protection conveyed by the anti-sense DAP3 RNA. This specific fragment of DAP3 may therefore compete with the endogenous DAP3 along the apoptotic pathway.

Interestingly, the dominant-negative effects of Δ230 mutant depended completely on the presence of a functional P-loop motif. The same deletion in the P-loop mutant of DAP3 completely lost its death-protective effects. When assayed by co-transfections with p55/Fas, the percentage of apoptotic cells among the GFP positive cells remained high (~85%), as in the control transfections (Figure 5A). This was not due to differential stability of the two deletion mutant proteins as they are expressed at similar levels (not shown). It is concluded, therefore, that the Δ230 deletion mutant of DAP3 acts in a dominant-negative fashion and inhibits apoptosis induced by Fas in a manner that depends on the integrity of the P-loop motif. It is also shown that the deletion of an additional 53 amino acids from the C-terminus of Δ230 mutant (Δ177) eliminated its death-protective features (Figure 4A).

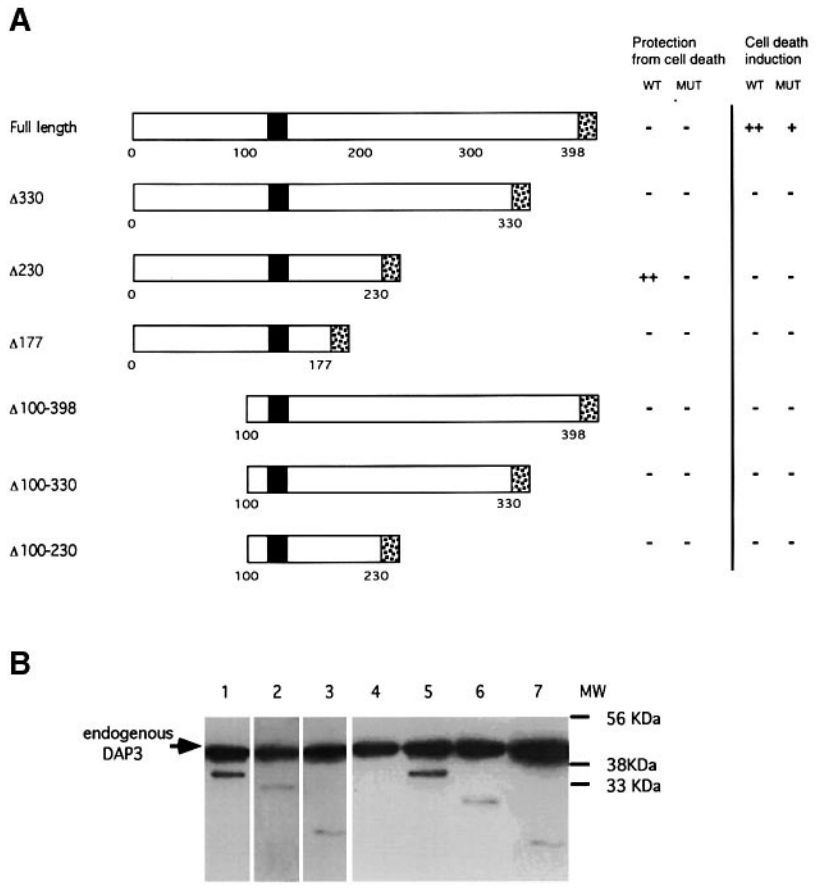

Fig. 4. DAP3 deletion mutants. (**A**) Schematic representation of the various deletions made in the DAP3 protein (wild type, WT; P-loop mutant, MUT). Numbers represent the amino acid sequence. The P-loop is represented by the black box, the dotted box represents the FLAG epitope. All the constructs were assayed in 293 cells for cell death induction (as in Figure 2) or for protection from Fas-induced cell death (as in Figure 5A). (**B**) Expression of the various deletion mutants in 293 cells: lane 1, Δ330; lane 2, Δ230; lane 3, Δ177; lane 4, full-length DAP3; lane 5, Δ100–398; lane 6, Δ100–330; lane 7, Δ100–230. Endogenous DAP3 is indicated by an arrow. The Western blot was probed with anti-DAP3 antibodies.

DAP3 is also involved in TNF-α-induced apoptosis

To test whether DAP3 might be involved in the mediation of TNF-α-induced cell death, the dominant-negative acting form of DAP3 was co-transfected with p55-TNF-R1 into 293 cells. As shown in Figure 5B, transfection of the p55 TNF-R into 293 HEK cells resulted in massive cell death by 24 h. Co-transfection of p55-TNF-R1 together with the dominant-negative acting form of MORT-1/FADD (DN-MORT-1), used again as a positive control for effective cell death protections, reduced the number of apoptotic cells to ~10%. The dominant-negative form of DAP3, the Δ230 mutant, protected the transfected cells from p55-TNF-R1-induced cell death, whereas the P-loop mutant form of the Δ230 fragment of DAP3 was severely impaired in its ability to protect from apoptosis (Figure 5B). Thus, the dominant negative form of DAP3 can protect cells from the p55-TNF-R1-induced cell death. This implicates DAP3 as a positive mediator of TNF-α-induced cell death.

Interestingly, the protections conveyed by the Δ230 DAP3 mutant did not require newly synthesized proteins, since they also occurred in the presence of the protein synthesis inhibitor cycloheximide. In these experiments, apoptosis was induced by addition of TNF-α ligand, together with cycloheximide at 24 h after transfections with death-protective constructs expressing DN-MORT-1 and Δ230 DAP3. As shown in Figure 5C, the Δ230 mutant was capable of reducing the number of apoptotic cells in the presence of cycloheximide from 55 to 22%, suggesting that DAP3 itself is part of the main death pathway, which does not require *de novo* protein synthesis for its function.

Functional position of DAP3 with respect to the death inducing signaling complex (DISC) formation and terminator caspases

In order to place DAP3 along the apoptotic pathways of TNF-α and Fas, several known components of the system were assayed in co-transfection assays. First, the functional position of DAP3 was determined with respect to components from the DISC. To this aim, it was tested whether the dominant-negative form of MORT-1/FADD (DN-MORT-1) and the dominant-negative form of caspase-8 might rescue the DAP3-induced cell death in 293 cells. These constructs efficiently block cell death initiated by activation of Fas and TNF receptors as they interfere with the formation of the active signaling complex at the receptor level (Boldin *et al*., 1997; Figure 5A and B). Neither DN-MORT-1 nor the dominant-negative form of caspase-8 suppressed cell death induced by DAP3 (Figure 6), suggesting that they may function upstream of DAP3. In addition, the components of the DISC, recruited to Fas receptors after their stimulation with agonistic antibodies, were immunoreacted with anti-DAP3 antibodies following 2-D gel fractionations and immunoblotting. These experiments showed clearly that DAP3 is

definitely not a member of the DISC (P.H.Krammer, personal communication).

Next, the possibility that other caspases may function as downstream mediators of DAP3 was tested using two broad-range caspase inhibitors: crmA, which is a cowpox virus protein, and p35, which is a baculovirus-encoded protein. These inhibitors were shown to inhibit the proteolytic activity of several caspases, and as a consequence to block TNF-α- or Fas-induced cell death (Hardwick, 1998). Co-transfection of DAP3 with either one of these inhibitors in 293 cells decreased cell death significantly (Figure 6). These results functionally place some members of the caspase family, other than caspase-8, downstream to DAP3, along pathways leading to cell death.

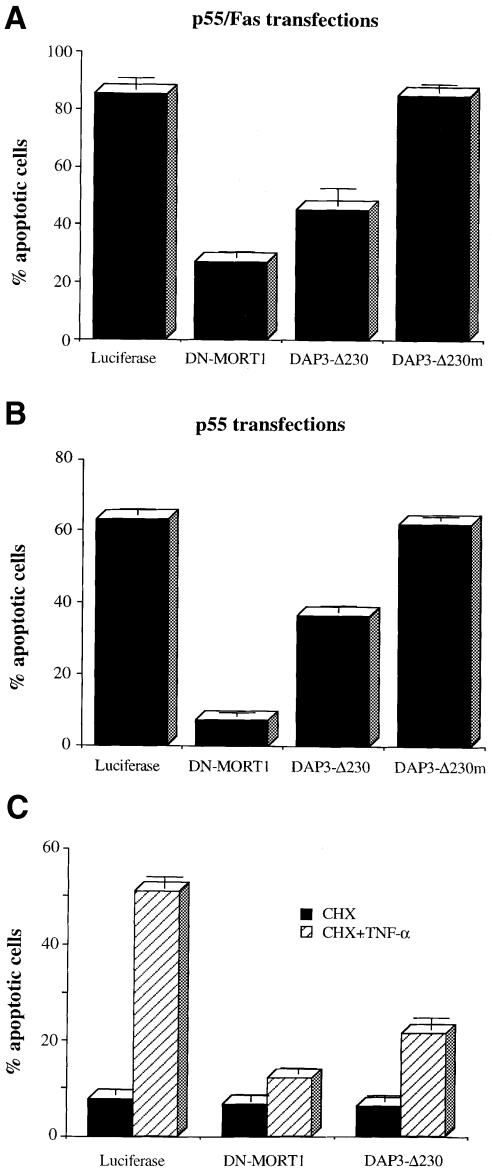

Fig. 5. Expression of the Δ230 deletion mutant of DAP3 protects 293 and HeLa cells from Fas and TNF-α-induced apoptosis. (**A**) Transient transfection of 293 cells with vectors encoding GFP, Fas/TNF chimera, and either a dominant-negative form of MORT-1/FADD (DN-MORT-1), or Δ230 or Δ230m (P-loop mutant). The number of apoptotic cells was scored using fluorescence microscopy. The results represent the average of four independent transfections performed in triplicate. (**B**) Transient transfection of 293 cells with vectors encoding GFP, p55-TNF-R1, and either a dominant-negative form of MORT-1/FADD (DN-MORT-1), or Δ230 or Δ230m (P-loop mutant). Scoring of apoptotic cells was performed as in (A). (**C**) Transient transfection of HeLa cells with vectors encoding GFP and either luciferase or a dominant-negative form of MORT-1/FADD (DN-MORT-1) or Δ230. Cells were treated 24 h post transfection with a combination of TNF-α and cycloheximide. The number of apoptotic cells was scored under fluorescent microscopy 2 h post treatment. The results represent the average of three independent transfections done in triplicates.

Identification and cloning of a C.elegans homolog of DAP3

By searching the EMBL protein databases with a BLAST algorithm (Altschul, 1990) using the full-length DAP3 cDNA as the query sequence, clone cec14a_4.2 was retrieved as a high scoring hit. The cec14a_4.2 clone, which is localized to chromosome II, is actually a potential open reading frame (ORF) predicted from *C.elegans* genomic DNA by the 'ProteinPredict' program (Wilson *et al*., 1994). No related expressed sequences were available which further prompted the cloning project. The cec14a_4.2 was predicted to encode a 'putative' protein of 774 amino acids whose N-terminus half shares high homology with the entire DAP3 protein, while the C-terminus differs completely (Figure 7A and B). To test whether the *C.elegans* DAP3 homolog is indeed an expressed gene, Northern blot analysis and PCR analysis of a *C.elegans* mixed-stage cDNA library (Okkema, 1994) was performed. Using primers from the most conserved exon at the DNA level (exon 2 according to the ProteinPredict program), and the cDNA library as the template, a single polymerase chain reaction (PCR) product of the expected size was produced, indicating that the cec14a_4.2 is indeed transcribed and present in the cDNA library (not shown). Using the PCR product as a probe for Northern analysis, a single mRNA product, corresponding in size to 1.3 kb, was identified (not shown). The size of the mRNA matched the human DAP3 mRNA, and contradicted the prediction of a longer transcript coding for a 774-amino-acid protein product.

Upon screening a *C.elegans* mixed-stage cDNA library with the same PCR-amplified DNA fragment, several

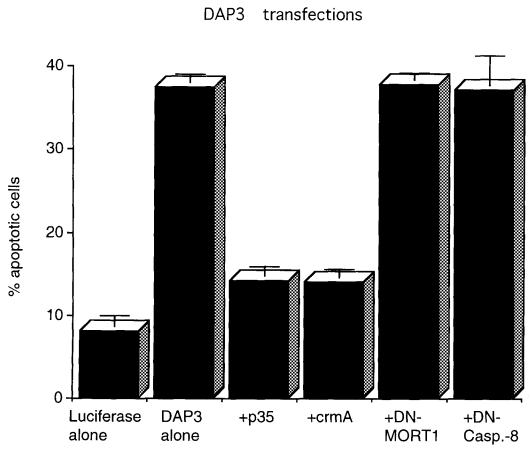

Fig. 6. DAP3-induced cell death in cells co-transfected with caspase inhibitors.Transient transfection of 293 cells with vectors encoding GFP, DAP3 and either dominant-negative forms of MORT-1/FADD (DN-MORT-1) or caspase-8 (DN-Casp.-8), or p35 or crmA. The number of apoptotic cells was scored using fluorescence microscopy. The results represent the average of four independent transfections performed in triplicate.

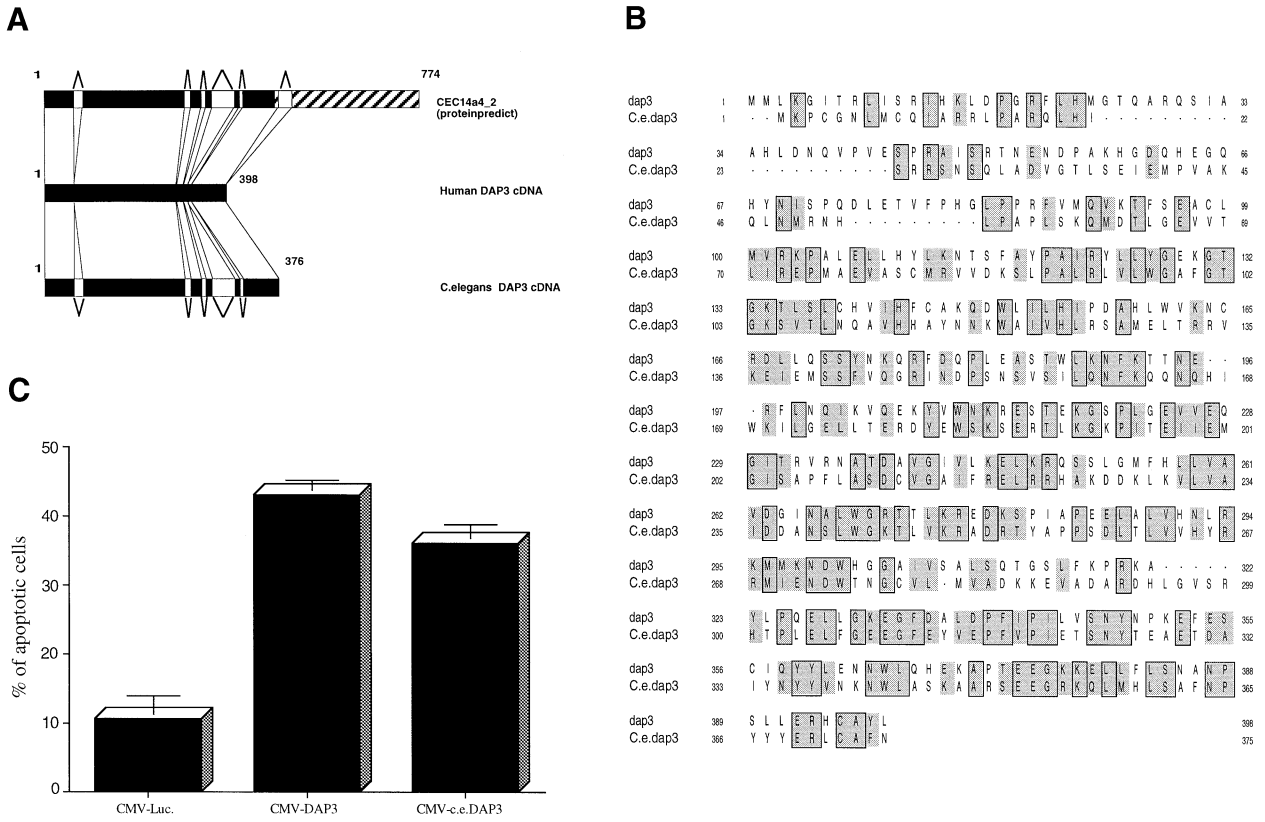

Fig. 7. Sequence and genomic organization of a DAP3 homolog in *C.elegans*. (**A**) Genomic organization of DAP3 in *C.elegans* as predicted by the ProteinPredict program and as determined experimentally. Exons coding for high homology sequences are in black, non-homologous sequences are striped and introns are white. Numbers refer to amino acids. (**B**) Comparison of the DAP3 human and nematode protein sequences, determined by direct sequencing of isolated cDNA. Identities are blocked and similarities are shaded. (**C**) Overexpression of c.e.DAP3 in HeLa cells induces cell death. Transient co-transfection of GFP and either CMV–DAP3 or CMV–c.e.DAP3 into HeLa cells. Numbers of apoptotic cells was scored by visualization with fluorescence microscopy, as in Figures 2 and 5. The results represent three different transfections, each performed in triplicate.

clones were isolated, among which the four longest clones with inserts of 1.3 kb in size, were selected for further analysis. All four clones had the same insert of 1309 nucleotides, comprising a single ORF spanning the first six exons (Figure 6A). Thus, the prediction of Protein-Predict was wrong in that it spliced a seventh exon that is not part of DAP3 but rather of an adjacent gene. The actual product of cec14a_4.2 is a 1.3 kb transcript that codes for a protein of 376 amino acids. Alignment of the human and *C.elegans* DAP3 proteins shows 35% identity and 64% similarity between the two proteins (Figure 7B). The homology spans over the entire protein with no preference for any specific region.

The C.elegans DAP3 shares cell death-promoting features with the human DAP3

In light of the significant structural homology between *C.elegans* DAP3 gene and the human DAP3, it became of interest to test whether the nematode gene still possesses the cell death-promoting functions, characteristic of the human protein when assayed in mammalian cells. To test this possibility, we co-transfected HeLa cells with GFP and either human CMV–DAP3 or *C.elegans* CMV–DAP3 (c.e.DAP3) and compared the numbers of apoptotic cells by fluorescence microscopy. Ectopic expression of c.e.DAP3 resulted in an average of 36% apoptotic cells, 24 h post transfection. This is slightly lower than the effect of human DAP3 in these transfections (statistically significant at $\alpha = 0.05$), which gave an average of 43% apoptotic cells at the same time point (Figure 7C).

Discussion

DAP3 is a mediator of Fas and TNF-α-induced cell death

We have shown previously that the DAP3 gene is a positive mediator of IFN-γ-induced cell death (Kissil *et al*., 1995). This gene was isolated via an anti-sense knock-out screen designed to identify positive mediators of IFN-γ-induced cell death. Expression of anti-sense RNA from DAP3 cDNA fragment was able to protect HeLa cells from the apoptotic effect of IFN-γ. Here we demonstrate that DAP3 not only mediates the effect of IFN-γ, but also the effects of Fas and TNF-α, thus indicating that DAP3 is a mediator of cell death common to various types of apoptotic stimuli. The finding that DAP3 is a positive mediator of Fas-induced cell death was supported by two independent approaches. First, the HeLa polyclonal populations that were transfected with the DAP3 anti sense cDNA were protected when exposed to the anti-Fas agonistic antibody which induces apoptosis. Secondly, a panel of DAP3 deletion mutants was screened for the ability to protect 293 cells from the effect of Fas-induced cell death. With this aim we set up a system in which we transfected 293 HEK cells with a p55/Fas-fused cDNA. The latter expresses a chimeric protein composed of

the intracellular domain of the Fas receptor and the extracellular domain of the TNF-α receptor to achieve better receptor trimerization, independent of Fas ligand or antibody (Boldin *et al*., 1997). As shown, the transfection of the 293 cells with p55/Fas led to massive cell death at 24 h post transfection. Co-transfection of the p55/Fas, together with various deletion mutants of DAP3, revealed that a certain DAP3 fragment, Δ230 mutant, seems to act in a dominant-negative fashion and protected the transfected cells from Fas-induced apoptosis. Thus, both approaches demonstrated independently that DAP3 is a positive mediator of Fas-induced apoptosis. It was also tested here whether DAP3 might mediate TNF-α-induced apoptosis. Thus, again we used 293 cells and transfected them with the p55-TNF-R1 receptor, which induces apoptosis when overexpressed (Boldin *et al*., 1997). The co-transfection of p55-TNF-R1 with the Δ230 form of DAP3-protected cells from the effect of this activated receptor as well.

Recent work has shown that apoptotic signaling molecules such as TNF-α induce both death-inducing and protective pathways simultaneously (Wallach *et al*., 1998). DAP3 could exert its positive effects on apoptosis either by being an intrinsic component of the death pathway or alternatively by suppressing the protective branch of the signal. Protective pathways in some of the well-studied cases, such as the TNF-α-induced NFκB pathway, were clearly shown to be protein synthesis dependent. Yet, the Δ230 mutant of DAP3 continued to be effective in suppressing TNF-α-induced cell death in the presence of cycloheximide, indicating that it is competing with a component belonging to the cell death induction branch of the signal. Similar results were obtained when agonistic anti-Fas antibodies were added together with cycloheximide (not shown). This would indicate that DAP3 is a positive mediator of cell death and not a suppressor of the protective arm of signaling.

By the use of viral produced inhibitors of caspases, we showed that members of this family of cysteine proteases are involved in DAP3-induced cell death. Among the two inhibitors that were used, crmA is believed to be more specific to the sub-family of the ICE-like proteases, whereas p35 has a wider spectrum of inhibition (reviewed in Villa *et al*., 1997). In our experiments, both inhibitors suppressed DAP3-induced cell death to a similar extent. These results suggest that ICE-like proteases mediate the effect of DAP3. The caspase family in general, and the ICE-like subgroup in particular, include several proteases acting at different positions along death pathways; they may belong either to the initiator or executioner type of caspases. Therefore, it is hard to speculate at the present time about the specific proteases that mediate the effect of DAP3.

The dominant-negative forms of either FADD/MORT-1 or caspase-8 did not inhibit DAP3-induced cell death. This result, and the finding that DAP3 is not localized to the DISC (P.H.Krammer, personal communication), indicates that DAP3 functions downstream to the signaling complex formed at the receptor level. This fits with our finding that the protection from cell death resulting from interfering with the expression/function of DAP3 was always more modest than those obtained by blocking components of the DISC. Therefore, DAP3 probably functions further downstream past a point of bifurcation of the cell-death signal.

Structural and functional analysis of the DAP3 protein

Two main aspects of DAP3 were studied with regard to function and structure. The fact that a potential P-loop motif exists in DAP3 might implicate it as a nucleotide binding protein. A mutation that has been shown to abolish the nucleotide binding capabilities of various proteins was introduced into DAP3. This did not effect the overall stability of the protein as it was expressed to similar levels of the wild-type DAP3. It did, however, impair its function as an inducer of cell death when overexpressed. This impairment reduced the effect of DAP3 by >2-fold. It seems safe to say that the integrity of the P-loop is essential for DAP3 to exert its full effect. This is also the case in the dominant-negative form of DAP3, Δ230. The insertion of the point mutation impedes the full effect of the dominant-negative protein without effecting its expression levels. This is not surprising in light of the basic assumption which predicts that some of the biochemical functions of the full-length protein are retained in the dominant-negative form of a protein and are essential for its ability to compete with the endogenous gene. While the functional importance of the P-loop motif in DAP3 is well documented in this work, the biochemical proof as to the nature of the nucleotide that it binds (e.g. ATP or GTP) still awaits further experimentation.

The deletion mapping was a second approach to attempt to elucidate regions of the DAP3 that might be important for its function. As can be seen from the right panel in Figure 4, all deletions from DAP3 rendered it incapable of cell-death induction when overexpressed. Thus, within the resolution of our experiments, the full length of DAP3 is required for induction of cell death by overexpression. A truncated protein ranging from amino acids one to 230 is capable of acting in a dominant-negative fashion in blocking Fas- and TNF-α-induced cell death, suggesting that some of the protein's functional domains reside in this fragment. This property is lost as soon as additional deletions are made at either end of the protein.

Caenorhabditis elegans DAP3

In contradiction to the ProteinPredict program, we have found by cDNA cloning that the actual nematode sequence does not splice at the seventh intron, thus producing a 376-amino-acid protein (Figure 7A). This is in accordance with the size of the transcript observed by Northern blot analysis (1.3 kb) and actually shows greater homology to the human DAP3 protein at the extreme C-terminus (Figure 7B).

At the functional level, the overexpression of the *C.elegans* DAP3 cDNA in HeLa cells induced cell death at levels slightly lower than those of human DAP3. This indicates that the worm protein is not only evolutionary conserved at the structural level, but is also conserved in its function. However, this does not mean automatically that the *DAP3* gene is involved in programmed cell death in *C.elegans*. It is quite possible that this gene participates in some other highly conserved functions common to worms and mammals, and that the involvement of DAP3 in mammalian cell apoptosis is an acquired function occurring as a later evolutionary event. Experiments con-

sisting of *DAP3* knock out in *C.elegans* must be conducted to assess directly the issue of whether the loss of this gene results in defects in the process of programmed cell death in nematodes.

Materials and methods

Cell lines and transfections

HeLa and 293 HEK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transient transfections of 293 HEK and HeLa cells were performed by the calcium phosphate method as described by Kissil *et al*. (1995). 293 cells were seeded the previous night at a density of 10^5 cells/well in 6-well plates, and then treated with a transfection mix containing 0.5 μg GFP, 0.5 μg p55/Fas or p55-TNF-R1 and 1.5 μg of MORT-1/FADD or the various deletion mutants. The HeLa cells were transfected as above with 0.5 μg GFP, 1.5 μg Δ230 and 0.5 μg of CD95/Fas.

Stable transfections of HeLa cells with vectors expressing the DAP3 antisense were performed as follows: cells were plated in 9 cm plates at 10^6 cells/plate the night before and then transfected the next day with 20 μg plasmid DNA per plate. The polyclonal HeLa cell populations expressing anti-sense DAP3 RNA (#21, #22), as described previously by Kissil *et al*. (1995), were grown in the continuous presence of hygromycin B (200 μg/ml).

Cell death assays

Fas-induced cell death in the stable HeLa transfectants was performed in 96-well plates seeded at a density of 2×10^4 cells/well the night before. The cells were treated for 40 h with 25 U/ml of IFN-γ to raise levels of Fas in the HeLa cells (Itoh *et al*., 1991). The anti-Fas agonistic antibody (IgG3; gift of P.H.Krammer) was added at 50 ng/ml. Cell viability was scored 28 h after the addition of the antibody using a neutral dye uptake assay as described previously (Wallach, 1984).

Cell death via p55-TNF-R1 and p55/Fas overexpression was scored at 24 h post transfection. Only transfected cells were scored by fluorescence microscopy. The TNF-α- and Fas-induced cell death, assayed in transiently transfected HeLa cells in the presence of cycloheximide, was performed by treating the cells, 24 h after transfection, with 10 μg/ml cycloheximide and either 2×10^3 U/ml of TNF-α (R&D) or 25 ng/ml of the anti-Fas agonistic antibody. Transfections included 0.5 μg GFP, 1.5 μg Δ230 and 0.5 μg CD95/Fas (to increase the response to anti-Fas antibodies in these cells). Cell death was determined on basis of the morphology of transfected cells, i.e. detachment from plate, membrane blebbing and cellular fragmentation. All experiments represent results from at least three different transfections, each performed in triplicate.

FACS analysis of REF

Primary REFs were prepared as described (Finlay, 1995). Cells (10^6) were transfected with 5 μg of the indicated plasmids (pCDNA3 or pCDNA3-DAP3) together with 0.5 μg of DNA coding for farnesylated-GFP. The transfection was performed using Fugene reagent (Boehringer Mannheim). Forty-eight hours post-transfection, cells were harvested for analysis. Cells were washed once in cold phosphate-buffered saline (PBS), fixed in 5 ml of cold methanol (–20°C) for 30 min, centrifuged and resuspended in 0.5 ml PBS containing RNase A (50 μg/ml) and PI (100 μg/ml). Cells were then subjected to flow cytometric analysis, where GFP-positive cells were gated by their high fluorescent intensity (FL1). For each sample, 5000 GFP-positive cells were collected and cell-cycle distribution was analyzed according to DNA content using PI staining. The results represent three independent transfections.

Construction of plasmids and mutations

The DAP3 gene was cloned into pcDNA3 (Invitrogen), downstream to a CMV promoter with a FLAG epitope (Kodak, IBI) fused to C-terminal end of the protein in the *Eco*RI and *Xho*I sites. Site-directed mutagenesis at nucleotides 473–475, changing AAA to GCA (thus changing the amino acid sequence from K to A) was performed as described (Sambrook *et al*., 1989). All deletion mutants were created by PCR with the pcDNA3-DAP3 (wild type or mutant) as template and the T7 as the 5′ primer. The various deletions were made from the 3′ by PCR with a primer ending with a sequence coding for a FLAG-epitope and a stop codon (sequences will be supplied upon demand). All deletion mutants are in pCDNA3 in the *Eco*RI–*Xho*I sites. The N-terminus 100-amino-acid deletion was created by cutting the various deletion mutants with *Hin*dIII and religating. The *C.elegans* DAP3 cDNA was cloned by PCR, with a FLAG-epitope at the C-terminus of the protein, into the pCDNA3 *Bam*HI and *Xho*I sites. The plasmids expressing DN-MORT-1, MACHα-C360S (DN-Casp.-8), p55-TNF-R1, CD95/Fas and p55/Fas are described in Boldin *et al.* (1997).

Northern blot analysis

Total RNA was extracted from *C.elegans* mixed stages using Trireagent (MRC). Samples of 20 μg total RNA or 5 μg poly(A)$^+$ RNA were separated on 1% agarose gels and hybridized to Hybond-N nylon membranes (Amersham) as described previously (Sambrook, 1989). DNA probes were prepared using [α-^{32}P]dCTP with commercially available random priming kits (Boehringer Mannheim). Prehybridization, hybridization and washing of filters was performed as described previously (Kissil *et al*., 1995). The blots were exposed for as long as indicated on X-ray film (Kodak).

Western blot analysis and antibodies

293 cells were harvested and protein extracts were prepared as previously described (Deiss *et al*., 1995). The protein samples (50 μg/lane) were fractionated by SDS–PAGE on a 10% gel. The proteins were transferred to a nitrocellulose filter (Schleicher and Schuell) with a semi-dry semiphor blotter (Hoefer Scientific Instruments). The blots were reacted either with anti DAP3 monoclonal antibody (Transduction Laboratories, Lexington, KY) or anti-FLAG antibodies (Kodak). Immunodetection was performed using the ECL detection system (Amersham).

Isolation and sequence analysis of full-length C.elegans DAP3 cDNA

The human and *C.elegans* DAP3 sequences were used to search the databases, applying FASTA (GCG package) at the nucleotide level, and FASTS, BLASTP and BLOCKS at protein level. The MOTIF program (GCG) was used to analyze the sequence for potential motifs.

Using primers from the second predicted exon of the *C.elegans* gene and *C.elegans* genomic DNA as template, a probe with the expected size of 350 bp was generated. The same PCR conditions and primers were used to screen a mixed-stage cDNA library as template (Okkema, 1994) for existence of the DAP3 sequences. Radiolabeled probe was used to screen the λgt10 mixed-stage cDNA library. Approximately 10^6 plaque forming units (PFUs) were screened with the above mentioned cDNA insert, and four positive clones were isolated. Plaque hybridization was performed under stringent conditions. One clone, carrying the longest cDNA insert was chosen for further work. The insert was subcloned by PCR with λgt10 primers into a pGEM-T vector (Promega). Sequencing of both strands was done by primer walking, fully automated on an Applied Biosystems DNA sequencer 373A.

The DDBJ/EMBL/GenBank accession No. of *C.elegans* DAP3 is AF058329.

Acknowledgements

We thank D.Wallach for providing expression vectors carrying the p55, p55/Fas chimera, CD95/Fas, MACHα-C360S and DN-MORT-1, C.Kahana for the GFP expression vector, P.H.Krammer for anti-APO-1 agonistic antibodies, T.Hunter for farnesylated GFP and P.Okkema for the *C.elegans* cDNA library. This work was supported by the Israel Foundation, which is administered by the Israel Academy of Science and Humanities, and by QBI Ltd. A.K. is the incumbent of the Helena Rubinstein Chair of Cancer Research.

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Received May 13, 1998; revised June 23, 1998; accepted November 24, 1998