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Isolation of *DAP3*, a Novel Mediator of Interferon-γ-induced Cell Death*

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Interaction of certain cytokines with their corresponding cell-surface receptors induces programed cell death. Interferon-γ induces in HeLa cells a type of cell death with features characteristic of programed cell death. Here, we report the isolation of a novel gene, *DAP3* (death-associated protein-3), involved in mediating interferon-γ-induced cell death. The rescue of this gene was performed by a functional selection approach of gene cloning that is based on transfection with an antisense cDNA expression library. The antisense RNA-mediated inactivation of the *DAP3* gene protected the cells from interferon-γ-induced cell death. This property endowed the cells expressing it with a growth advantage in an environment restrictive due to the continuous presence of interferon-γ and thus provided the basis of its selection. The gene is transcribed into a single 1.7-kilobase mRNA, which is ubiquitously expressed in different tissues and codes for a 46-kDa protein carrying a potential P-loop motif. Ectopic expression of *DAP3* in HeLa cells was not compatible with cell growth, resulting in a 16-fold reduction in the number of drug-resistant stable clones. The data presented suggest that *DAP3* is a positive mediator of cell death induced by interferon-γ.

Programed cell death is a highly controlled process required for the normal development, maintenance, and survival of the multicellular organism. Among the physiological signals that induce inhibition of cell proliferation and cell death are diffusible polypeptides such as interferons, tumor necrosis factors, and transforming growth factor-β (1). To date, relatively few of the positive mediators of cell death in mammalian systems have been identified and characterized. These include *p53*, interleukin-1β-converting enzyme, *nedd2*/ICH_L, *CPP32*, *nur77*, *BCL-Xs*, *BAX*, and *granzyme B* (2–11). Recently, a functional approach of gene cloning developed in our laboratory was used, as previously reported, to rescue genes that mediate IFN-γ[1]-induced cell death (12). The effect of IFN-γ in epithelial cells is biphasic. The first phase consists of cell proliferation arrest, which is subsequently followed by a second phase of cell death, which has the characteristics of programed cell death, including cell shrinkage, membrane blebbing, and condensation and fragmentation of nuclear chromatin (13). The approach developed, termed technical knockout (TKO), is based on random inactivation of genes by transfection of HeLa cells with an antisense cDNA expression library, cloned into an Epstein-Barr virus-based episomal vector (pTKO1). The HeLa cells were then exposed to the selective pressure of IFN-γ over a 4-week period. At the end of the selection period, only cells that were protected from the effect of IFN-γ had survived.

The antisense cDNA clones rescued from these cells were tested in secondary transfections for their ability to reduce the susceptibility of HeLa cells to IFN-γ. Those that were positively scored were sequenced, and novel fragments were then used to probe a full-length cDNA library. Out of the genes rescued, by this method, one turned out to be the gene coding for thioredoxin (12). Recently, two other novel genes have been described. The first, *DAP1* (death-associated protein-1), is a basic, proline-rich 15-kDa protein. The second, *DAPk* (death-associated protein kinase), is a novel Ca^{2+}/calmodulin-dependent serine/threonine kinase that carries eight ankyrin repeats and a "death" domain (13, 14). In this work, we describe the isolation and characterization of a fourth gene, *DAP3* (death-associated protein-3).

MATERIALS AND METHODS

Construction of pTKO1-based cDNA Antisense Expression Library—The construction of the HeLa cDNA library and cloning into pTKO1 vector were performed as described (12).

Rescue of Antisense cDNAs and Transfections—Transfection of the antisense cDNA library into HeLa cells was done by the standard calcium phosphate technique. The pTKO1 vectors carrying antisense cDNA fragments were isolated as described (12, 13). Two secondary polyclonal populations, expressing the *DAP3* antisense cDNA *Hin*dIII-*Bam*HI fragment, were generated by secondary transfection of subconfluent monolayers of HeLa cells with 40 μg of the corresponding plasmid (named pTKO1-259). Pools of 10^4 independent stable clones were generated from each transfection. Transfectants were maintained as stable polyclonal populations with 200 μg/ml hygromycin B (Calbiochem).

Isolation and Sequence Analysis of Full-length DAP3 cDNA—A radiolabeled cDNA insert from pTKO1-259 was used to screen a λgt10 amplified cDNA library from K562 cells. Approximately 2×10^6 plaque-forming units were screened with the 259 cDNA insert, and 10 positive clones were isolated. Plaque hybridization was performed under stringent conditions. One clone, carrying the longest sense cDNA insert, was chosen for further work. The insert was subcloned into a Bluescript vector (Stratagene). Sequencing of both strands was done by primer walking, fully automated on an Applied Biosystems DNA 373A sequencer.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X83544.

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[1] The abbreviations used are: IFN-γ, interferon-γ; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame.

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The sequence was used to search the data bases, applying FASTA (Genetics Computer Group package) at the nucleotide level and FASTS, BLASTP, and BLOCKS at the protein level (15, 16). The MOTIF program (Genetics Computer Group software package) was used to analyze the sequence for potential motifs.

Neutral Red Dye Uptake Viability Assay—The different transfected polyclonal HeLa cell populations were grown in 96-well microtiter plates. The initial number of cells was 15,000/well, and the cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone Laboratories) and 1% glutamine. Treated cells were exposed to IFN-γ (1000 units/ml) over a 12-day period (human recombinant IFN-γ, PeproTech); fresh medium and IFN-γ were supplemented every 4 days. At the indicated days, viable cells were stained with neutral red (Sigma) as described previously (17). Dye uptake was measured in quadruplicate using an automated enzyme-linked immunosorbent assay reader at $\lambda = 540$ nm.

RNA Blot Preparation and Analysis—Total RNA was extracted from HeLa cells using Trireagent (Molecular Research Center Inc.) and from normal human tissue as described (18). Samples of 20 μg of total RNA or 5 μg of poly(A)$^+$ RNA were separated on 1% agarose gels and hybridized to Hybond-N nylon membranes (Amersham Corp.) as described (19). DNA probes were prepared using [α-^{32}P]dCTP with commercially available random priming kits (Boehringer Mannheim). Prehybridization, hybridization, and washing of filters were performed as described (20). The blots were exposed for as long as indicated on x-ray film (Eastman Kodak Co.).

Preparation of Anti-DAP3 Antibodies and Immunoblot Analysis—The antibodies were generated in two rabbits as described (21). The antigen injected was glutathione *S*-transferase-DAP3 fusion protein excised from the gel. Antiserum from the 3rd week after the last boost was depleted of the anti-glutathione *S*-transferase antibodies by passing the antiserum through CNBr-activated Sepharose beads (Pharmacia Biotech Inc.) coupled to glutathione *S*-transferase. Titration and specificity of antibodies were determined, after depletion, by Western analysis of recombinant DAP3. HeLa cells were harvested, and protein extracts were prepared as described previously (13). The protein samples (200 μg/lane) were fractionated by SDS-PAGE on a 10% gel. The proteins were transferred to a nitrocellulose filter (Schleicher & Schuell) with a semidry semi-phor blotter (Hoefer Scientific Instruments). The blots were reacted with each of the two different polyclonal antibodies, and immunodetection was done using the ECL detection system (Amersham Corp.).

Expression of DAP3 in Reticulocyte Lysate—The full-length cDNA insert cloned into pBluescript SK (Stratagene) was used as a template for *in vitro* transcription from the T7 promoter. RNA (1 μg) was translated in the reticulocyte lysate (Promega) with [^{35}S]methionine as a labeled precursor. The reaction products were resolved by separation on SDS-10% polyacrylamide gel. To amplify the signal, the gel was immersed for 60 min in 10% acetic acid, rinsed in double distilled water for 10 min, and immersed in salicylic acid (1 M solution) for 20 min. The gel was dried and exposed for 1 h to x-ray film (Kodak) at −80 °C with an intensifying screen.

Overexpression of DAP3 in HeLa Cells—The *DAP3* gene was cloned into pCDNA3 (Invitrogen) downstream of a cytomegalovirus promoter and transfected into HeLa cells (30 μg/5 × 10^5 cells) as described (13). Empty vector was used as a control. Two days after transfection, G418 was added to the medium (800 μg/ml). The different cells were grown for an additional 10 days, and at that time, the number of stable clones in the control transfections that were resistant to G418 was scored. Transfections with *DAP3* were scored after 3 weeks.

RESULTS

As described previously (13), HeLa cells were transfected with the antisense cDNA library, and selective pressure of both IFN-γ (750 units/ml) and hygromycin B (200 μg/ml) was applied over a 4-week period. The DNA plasmids extracted from the surviving clones were classified into six nonoverlapping groups according to cross-hybridization on Southern blots. The group of *DAP3* was composed of only a single member carrying a novel DNA fragment that was 252 base pairs long and was termed clone 259 (Table I in Ref. 13). The pTKO1-259 plasmid was then transfected in duplicate into HeLa cells, and two stable polyclonal cell populations were generated and maintained with hygromycin B (designated 259-t1 and 259-t2).

The effect of pTKO1-259 in protecting the cells from the

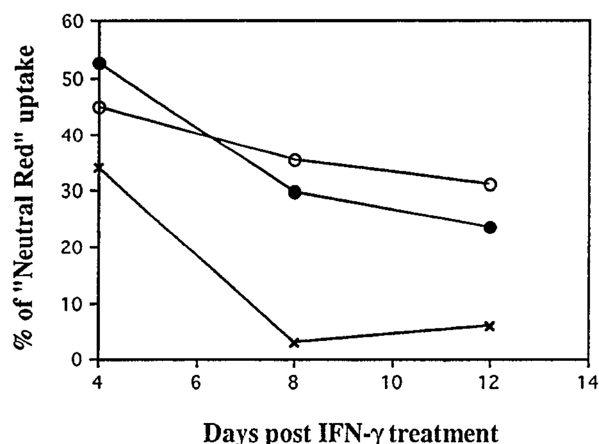

FIG. 1. ***DAP3* antisense RNA protects two HeLa populations from IFN-γ-induced cell death.** The fraction of viable cells was determined by comparing neutral dye uptake of IFN-γ-treated cells and nontreated cultures at the indicated time points. Control pTKO1-DHFR-transfected HeLa cells (×) and two independently pTKO1-259-transfected HeLa cells (●, ○) were used. The decline in neutral dye uptake on day 4 is due to the cytostatic effect of IFN-γ. Each point represents an average of a quadruplicate (S.D. ranging from 0.5 to 8%).

IFN-γ-induced antiproliferative and cell death effects was then examined. This was accomplished by comparing the residual cell viability, after IFN-γ treatment, between the antisense RNA-expressing cell populations and a nonrelevant polyclonal population of HeLa cells transfected with a pTKO1-DHFR control vector (12). The assay was performed by using neutral dye uptake into cells as a measure of viability (Fig. 1). During the first 4 days after exposure to IFN-γ, the cells stopped proliferating, but remained viable as previously detailed (13). This is due to the initial cytostatic effect of IFN-γ. During this period, all cell populations behaved in a relatively similar manner, as deduced from microscopic observations (data not shown) and from the similar values of decline in neutral red dye uptake. However, there was a clear difference in viability of the different cell populations from day 4 after IFN-γ treatment and later on. The pTKO1-DHFR-transfected cells displayed rapid and massive cell death, resulting in <5% cell viability on day 8 of IFN-γ treatment. The two polyclonal cell populations that expressed antisense RNA displayed, however, reduced susceptibility to the death-inducing effect of IFN-γ (~40% cell viability on day 8 of IFN-γ treatment). These data indicate that expression of antisense RNA from *DAP3* protects the HeLa cells exclusively from IFN-γ-induced cell death and not from its cytostatic action. The growth curves of 259-t1 and 259-t2 and of the pTKO1-DHFR-transfected cells, which were kept in the absence of IFN-γ, were indistinguishable (data not shown), suggesting that antisense RNA expression has no effect on the normal growth of the cells.

The pattern of RNA expression was then determined. The 252-base pair DNA fragment carried by pTKO1-259 hybridized on Northern blots to a single endogenous 1.7-kilobase mRNA transcript in the control HeLa cell cultures (Fig. 2*A*). In the 259-t1 cell culture, the antisense RNA was also detected, having the predicted size of 1 kilobase (comprising the 252 bases of the cDNA insert and 800 bases of sequence from the expression cassette) (12). The levels of antisense RNA exceeded by ~5-fold that of the endogenous *DAP3* sense mRNA. Addition of IFN-γ caused a further induction of antisense expression, bringing it to exceed the *DAP3* mRNA by 30-fold (Fig. 2*A*). This was due to the presence of an interferon-stimulated response element in the pTKO1 vector. The endogenous 1.7-kilobase mRNA did not appear to be influenced by IFN-γ; it appeared as a single transcript also when the full-length cDNA was used as a probe.

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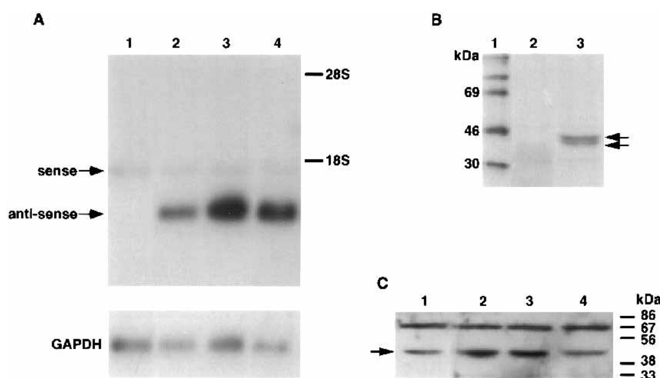

FIG. 2. **Expression of *DAP3* RNA and DAP3 protein.** *A*, total cellular RNA prepared from HeLa cells transfected with pTKO1-DHFR (*lane 1*) and from pTKO1-259-transfected cells that were treated with IFN-γ for 6 and 24 h (*lanes 3* and *4*, respectively) or left untreated (*lane 2*). Twenty micrograms of RNA were processed on Northern blots. DNA fragment 259 was used as a probe. The results were normalized according to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level. The filter was exposed overnight to x-ray film (Kodak) at −80 °C with an intensifying screen. *B*, *in vitro* translation of 1 μg of *DAP3* RNA, transcribed from the full-length cDNA clone (*lane 3*). *Lane 1*, molecular mass markers; *lane 2*, background obtained in the absence of RNA. The products were labeled with [^{35}S]methionine, separated by SDS-PAGE on a 10% gel, and exposed to x-ray film (Kodak) for 1 h at −80 °C with an intensifying screen. *C*, immunoblot analysis of DAP3 protein levels in control and protected cell populations treated with IFN-γ. *Lanes 1* and *2*, HeLa pTKO1-DHFR with no IFN-γ or 48 h after addition of IFN-γ to growth medium, respectively; *lanes 3* and *4*, cells transfected with pTKO1-259 with no IFN-γ or 48 h after treatment, respectively. Two bands are visible; the *upper* one (corresponding to a molecular mass of 67 kDa) is a nonspecific cross-reactivity of the antibodies, and the lower band (marked by an *arrow*) is that of DAP3 protein.

The antisense fragment was then used to screen a K562 cDNA library constructed in the λgt10 vector. A cDNA clone that spanned 1560 base pairs was chosen for sequencing. The sequence analysis of *DAP3* cDNA confirmed the antisense orientation of the *Hin*dIII-*Bam*HI insert of pTKO1-259 and the fact that it lies within the coding region of the *DAP3* mRNA (Fig. 3*A*). The sequence contains a single ORF starting at position 74 and ending at position 1268 with a stop codon. A polyadenylation signal (AATTA) is found at position 1517, and polyadenylation itself starts at position 1555 (Fig. 3*B*). The ORF codes for a potential protein of 398 amino acids and a calculated molecular mass of ~46 kDa.

A search of the data bases for possible homologies has not resulted in any significant matches. However, analysis of the protein sequence with the MOTIF program indicated that the protein contains a potential P-loop motif. This potential domain was identified through the consensus sequence (G/A)*XXXX*GK(T/S) at positions 128–135 and implies that DAP3 is a potential ATP/GTP-binding protein. No indications of the presence of a signal peptide or a transmembrane domain have been found (SAPS prediction).

An *in vitro* translation assay in the reticulocyte lysate confirmed the ORF prediction. The translation products of DAP3 cDNA were fractionated by SDS-PAGE to two closely migrating bands (Fig. 2*B*). The upper band corresponds to a protein of ~46 kDa. The lower, less abundant band is most probably due to alternative initiation of translation from a ATG codon that is in frame 24 amino acids downstream from the first ATG codon and hence represents a protein of ~42 kDa. This possibility is proposed since substitution of 2 base pairs upstream to the first ATG codon changed the ratio between these two bands in favor of the faster migrating form (data not shown).

The expression of DAP3 was then measured in HeLa cells using polyclonal antibodies made against the bacterially produced DAP3 protein. The antibodies recognize in HeLa cells a

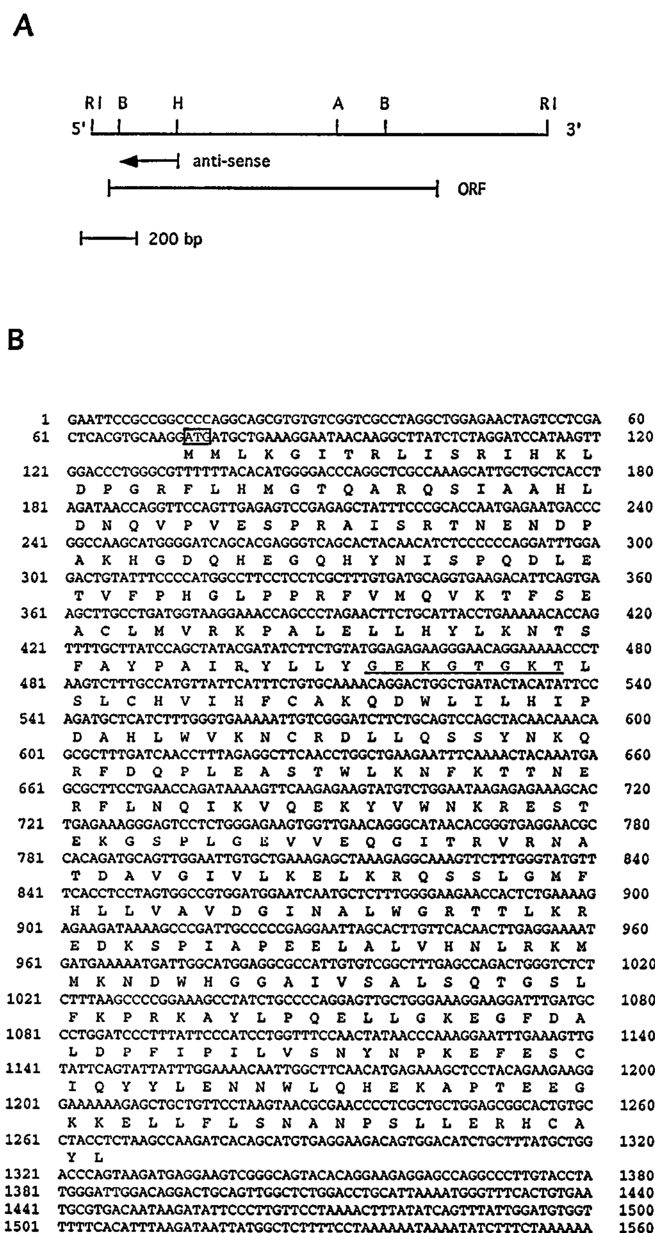

FIG. 3. **Nucleotide sequence of *DAP3* cDNA and deduced protein sequence.** *A*, shown are a schematic representation and limited restriction map of *DAP3* cDNA. *B*, *Bam*HI; *RI*, *Eco*RI; *H*, *Hin*dIII; *A*, *Ava*I. The ORF and the position of antisense fragment 259 are indicated. *B*, the nucleotide sequence of *DAP3* was determined for both strands. The deduced protein sequence is encoded below in one-letter code. The ATG initiation site is *boxed*, and the potential P-loop motif is *underlined*.

prominent band of 46 kDa, a mass consistent with the *in vitro* translated protein. In pTKO1-DHFR-transfected cells, DAP3 protein levels were elevated by ~2-fold at 48 h after addition of IFN-γ. In contrast, the 259-t1 cell culture, which expresses *DAP3* antisense RNA, displayed a 2–3-fold reduction in DAP3 protein levels 48 h after addition of IFN-γ to the growth medium (Fig. 2*C*). The high antisense RNA levels detected in the presence of IFN-γ therefore reduced the steady-state levels of the corresponding DAP3 protein by ~6-fold. As the *DAP3* antisense RNA did not seem to reduce endogenous *DAP3* mRNA levels, it seems likely that the antisense RNA exerts its effect by inhibiting, directly or indirectly, the translation of the *DAP3* mRNA transcript.

Since DAP3 is a mediator of IFN-γ cell death, it was plausible that elevated levels of the protein, by ectopic expression,

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Table I
Ectopic expression of DAP3 in HeLa is not compatible with continuous cell growth

Shown are the numbers of resistant colonies present on 9-cm plates counted at day 10 after addition of G418 (800 μg/ml) in the case of control clones and after 3 weeks of selection in the case of transfections with *DAP3*. Transfections were performed in triplicate.

Transfected vector	No. of G418-resistant colonies/plate
CMV[a]-*DAP3*	850
CMV-Neo	1.4×10^4

[a] CMV, cytomegalovirus.

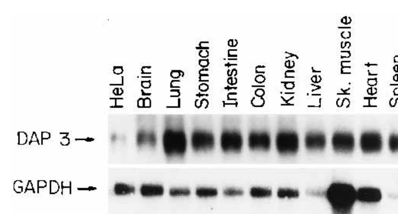

Fig. 4. **Tissue distribution of *DAP3* RNA transcript.** Five micrograms of poly(A)$^+$ RNA (18) were processed on a Northern blot. DNA fragment 259 was used as a probe. For quantitative analysis, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the *DAP3* mRNA densities. The blot was exposed for 1 h to x-ray film (Fuji) at -80 °C with an intensifying screen.

may cause cell death on its own. To answer this question, HeLa cells were transfected with *DAP3* under the control of the cytomegalovirus promoter and in parallel with a control vector, both carrying a gene conferring resistance to G418. Following transfection, G418 was added to the growth medium, and the number of colonies was subsequently scored (Table I). As evident from the data presented in Table I, ectopic expression of *DAP3* resulted in a 16-fold reduction in the number of G418-resistant HeLa colonies. Examination of the *DAP3*-transfected cells early after transfection (4–5 days, before mock transfectants are killed by the drug) revealed that a large portion of the transfectants rounded up and died. Moreover, at day 10, the colonies of the antibiotic-resistant stable cytomegalovirus-*DAP3* transfectants were much smaller than the colonies of the control transfectants and grew thereafter at a slow rate. These results were repeated in three independent transfections, each carried out in triplicate and with different preparations of plasmid DNA in each experiment.

In attempt to study the pattern of *DAP3* expression, the tissue distribution of *DAP3* mRNA was determined. A Northern blot containing mRNA from different human tissues was probed with the *DAP3* antisense cDNA fragment (Fig. 4). The results indicate that *DAP3* mRNA is ubiquitously expressed in all tissues analyzed. A 2-fold higher level of mRNA expression was found in lung tissue.

DISCUSSION

IFN-γ induces a type of cell death that has the characteristics of programed cell death. These features include chromatin condensation and segmentation, nuclear pyknosis, surface blebbing, budding off of cytoplasmic projections, and disappearance of surface microvilli (13). This is consistent with previous reports suggesting that IFN-γ has a role in the negative selection of T- and B-lymphocytes (22, 23). Hence, study of the genes mediating this effect should contribute to the elucidation of basic mechanisms underlying programed cell death.

To date, only a few positive mediators of cell death have been identified in mammalian systems. Some, like *p53* and c-*myc*, were well known for their other functions, and only later was their involvement in apoptotic cell death established (2, 24–26). Others, like *nur77*, were isolated by the subtractive hybridization approach of gene cloning and subsequently proved by functional assays to be indispensable in certain apoptotic systems (7, 8). The powerful genetic tools available in the nematode *Caenorhabditis elegans*, which led to the rescue of positive mediators of cell death such as *ced-3* and *ced-4*, were another starting point for the isolation of mammalian homologs. This led to the identification of interleukin-1β-converting enzyme as a potential mediator of cell death as well as to the recent isolation of other members of the interleukin-1β-converting enzyme/*ced-3* family (3, 5, 6, 27). Altogether, these findings demonstrate the importance of proteases in the process of cell death (28).

In this work, we demonstrate that, consistent with our previous reports, applying the functional approach of antisense knockout is a successful strategy for the isolation of novel genes that function as positive mediators of cell death. A growth advantage, conferred by expression of antisense RNA, serves as a strong positive selection measure in an environment restrictive due to the presence of IFN-γ. The isolation of *DAP3* as a mediator of IFN-γ-induced cell death is reported here. Transfection of cells with the antisense cDNA protected them from cell death in a clear manner, as judged by the nearly 10-fold enhanced viability in the continuous presence of IFN-γ. Yet, the antisense RNA did not have an effect on the cytostatic effect of IFN-γ, implying that these two processes are distinct.

Ectopic expression of *DAP3* alone caused massive cell death early after transfection and, as a consequence, a 16-fold reduction in the number of G418-resistant colonies. The ability of DAP3 to cause such a phenotype is consistent with its role as a mediator of cell death.

The single 1.7-kilobase mRNA of *DAP3* has a single ORF coding for a 46-kDa protein. This was confirmed by *in vitro* translation of the mRNA in the reticulocyte lysate. The mRNA is ubiquitously expressed in all tissues examined at comparable levels. This further suggests that the role of *DAP3* as a positive mediator of cell death is not restricted to certain tissues or inducers or, alternatively, may hint at additional, yet undefined functions. As the sequence of DAP3 shares no homology with known proteins, it is difficult at this stage to assign a role for *DAP3* in the process of IFN-γ-induced cell death. The potential P-loop motif, which is the only motif found in DAP3 by use of computer searches, was compared with the consensus P-loop domain in the seven classified families of ATP- or GTP-binding proteins (29). No similarity was found, and further work is needed to verify that this element is indeed a legitimate P-loop.

In our system, applying the approach of functional inactivation of genes has so far resulted in the identification of four different genes. One of these genes is the one coding for thioredoxin, a protein that reduces the intramolecular thiol groups that form disulfide bonds, suggesting that changes in the redox state of certain proteins are crucial for mediating IFN-γ-induced cell death (12). The other three genes identified so far code for novel proteins: *DAP1*, *DAPk*, and *DAP3*. This method of functional selection has proved fruitful in other systems as well. For instance, expression selection by the use of genetic suppressor elements was used to clone genes involved in mediating the effect of the anticancer drug etoposide (30). The recent identification and isolation of *Requiem*, which is a positive mediator of cell death induced by interleukin-3 deprivation, was based on a similar strategy (31). These data combine to demonstrate the feasibility of isolating positive mediators of cell death utilizing functional knockout approaches. Use of these approaches will no doubt enhance the understanding of the pathways underlying the mechanisms of cell death.

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