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Death associated proteins (DAPs): from gene identification to the analysis of their apoptotic and tumor suppressive functions

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The process of apoptosis (programmed cell death) has become the subject of intensive and extensive research over the past few years. Various approaches are being used to identify and study genes which function as positive mediators of apoptosis. Here, we address a novel approach of gene cloning aimed at isolating intracellular death promoting genes by utilizing a functional screen. This method, called TKO, was based on transfection of cells with an anti-sense cDNA library, followed by the selection of transfectants which survived in the continuous presence of a killing cytokine–interferon-γ. It led to the identification of five novel apoptotic genes and to the finding that a known protease–cathepsin D, is actively recruited to the death process. The five novel apoptotic genes (named DAP genes for: Death Associated Proteins) code for proteins which display a diverse spectrum of biochemical activities. The list comprises a novel type of calcium/calmodulin-regulated kinase which carries ankyrin repeats and a death domain (DAP-kinase), a nucleotide-binding protein (DAP-3), a small proline-rich cytoplasmic protein (DAP-1), and a novel homolog of the eIF4G translation initiation factor (DAP-5). Extensive studies proved that these genes are critical for mediating cell death initiated by interferon-γ, and in some of the tested cases also cell death induced by Fas/APO-1, TNF-α, and a detachment from extracellular matrix. Moreover, one of these genes, DAP-kinase, was recently found to display strong tumor suppressive activities, coupling the control of apoptosis to metastasis.

The advantage of functional approaches of gene cloning is that they select the relevant rate limiting genes along the death pathways in a complete unbiased manner. As a consequence, novel targets and unpredicted mechanisms emerged. A few examples illustrating this important point will be discussed. One relates to the calcium/calmodulin-dependent DAP-kinase, which is localized to the actin microfilaments. It was found that the correct localization of DAP-kinase to the microfilament network was critical for the execution of the apoptotic process, and more specifically for the disruption of the stress fibers–a typical hallmark of apoptosis. Another important breakthrough step in our understanding of apoptotic processes relates to the identification and analysis of the DAP-5 gene. The structure/function features of this novel translation regulator resemble the proteolytically cleaved eIF4G which appears in cells upon infection with some RNA viruses and which directs cap-independent translation. Thus, the rescue of DAP-5 highlighted the importance of regulation of protein translation in certain apoptotic systems. Finally, the isolation of cathespin D by our method suggests that lysosomal proteases are recruited during apoptosis, in addition to the well known caspase family of proteases, and that a unique pattern of regulation affecting the processing of this protease takes place. The major challenge now is to analyse how these diverse DAP gene activities constitute biochemical pathway(s) leading to programmed cell death, and what is their functional position with respect to other known positive mediators and suppressors of apoptosis such as the Bcl2 and caspase family members.

Keywords: apoptosis; DAP-kinase; cathepsin D; interferon-γ; metastasis; functional gene cloning

Introduction

Programmed cell death is a genetically controlled response of cells to commit suicide. The process, which displays distinctive morphological features, is highly conserved through evolution, and takes place in all nucleated animal cells. It is tightly controlled by environmental stimuli including extracellular diffusible factors, or membrane-bound molecules that mediate cell–cell or cell–matrix interactions, and by non-physiological insults such as genotoxic agents. This type of regulation allows the elimination of cells that were either produced in excess during development, have completed their role, are potentially deleterious to the organism, or have become seriously damaged. Programmed cell death is therefore a critical process during embryonic development, tissue remodeling, development of the immune system, and the control of tissue homeostasis (for reviews see Wyllie *et al*., 1980; Raff, 1992; Schwartz and Osborne, 1993). Several pathologies associated with the disruption of this fundamental process, have been characterized. While a decrease in the apoptotic rate is linked in some cases to abnormal expansion in cell number (e.g., in cancer or autoimmune diseases), an abnormal increase in the apoptotic rate is associated with some cell loss disorders (e.g. neurodegenerative diseases) (Thompson, 1995). The link to cancer takes place at the different stages of tumor development, one of which includes the loss of an early apoptotic checkpoint that safeguards cells against hyperproliferative oncogenic signals (Evan and Littlewood, 1998).

The spectrum of morphological hallmarks that has been attributed to the process is very wide, and includes specific nuclear alterations (e.g., chromatin condensation followed by its segmentation, internucleosomal DNA fragmentation), cytoplasmic condensation and/or vacuolization, disruption of cytoskeletal elements, cell surface blebbing, and in some cases,

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generation of apoptotic bodies that are rapidly phagocytosed by neighboring cells. The understanding of molecular mechanisms underlying these different cellular alterations has become, in the past few years, a major research goal in the field. The main challenge, in this respect, was to identify the genes that are components of the intracellular pathways which enable cells to receive, process and execute apoptotic signals from their environment. There has been an impressive progress in the past few years in understanding the intracellular molecule mechanisms of programmed cell death, emerging from studying both lower invertebrates and mammalian systems (see for examples Hengarthner and Horovitz, 1994; Steller, 1995; Thornberry and Lazebnik, 1998; Adams and Corry, 1998; Green and Reed, 1998). Yet, it is clear that many of the components functioning along the apoptotic pathways have not been discovered, which means that novel approaches and different insights should be introduced into this field. This paper will focus on one of these recently developed approaches which is based on a powerful genetic screen applied in mammalian cells. The novel molecular information obtained so far by this approach will be detailed, and future prospects will be discussed.

Hunting genes according to their function: the technical knock out (TKO) approach

The TKO strategy was designed with the intention of establishing a technology that will directly target functionally relevant genes involved in cell death, in a manner similar to the genetic screens used in *Drosophila melanogaster* or in *C elegans*. Instead of mutating the DNA, we directed our efforts at randomly inactivating gene expression by targeting the RNA, thereby bypassing the limitations of mammalian cells that lack the powerful genetic tools available in lower invertebrates. The targeting was performed in an unbiased manner by transfections with anti-sense cDNA expression libraries and producing a wide spectrum of complementary RNAs (Deiss and Kimchi, 1991). The assumption was that the specific antisense RNA-mediated inactivation of a rate limiting death-promoting gene would confer some growth advantage to cells that are continuously exposed to a killing agent. This advantage could then be used as a powerful forward selection to rescue the relevant cDNA. In other words, the genes of interest are selected and cloned by virtue of a defined phenotypic change – reduced susceptibility to death-inducing signals.

Interferon-γ (IFN-γ) was chosen as the preferred external agent which induces strong apoptotic responses in some target cells. In HeLa cells we found that this cytokine imposed a biphasic pattern of responses, i.e., a reversible proliferation arrest followed by programmed cell death (Deiss *et al*., 1995). This provided a unique system to study, within a single genetic constellation, the points where the cell cycle inhibitory and apoptotic mechanisms diverge. The cDNA library itself was designed to yield high levels of antisense RNAs by virtue of the fact that: the library was cloned into an EBV-based episomal vector which allows high copy number of episomes per cell; and the expression vector contained an IFN response element to further elevate the expression of antisense RNAs during the selection in the presence of IFN-γ (Deiss and Kimchi, 1991). The vector also included a resistance gene to the drug hygromycin B, thus eliminating the background of untransfected cells. In addition, the episomal nature of the vector allowed easier rescue of antisense cDNAs from surviving clones.

The rescue of cell-death protective cDNA fragments

As depicted in Figure 1, the cDNA library (prepared from a mixture of untreated and IFN-γ-treated HeLa

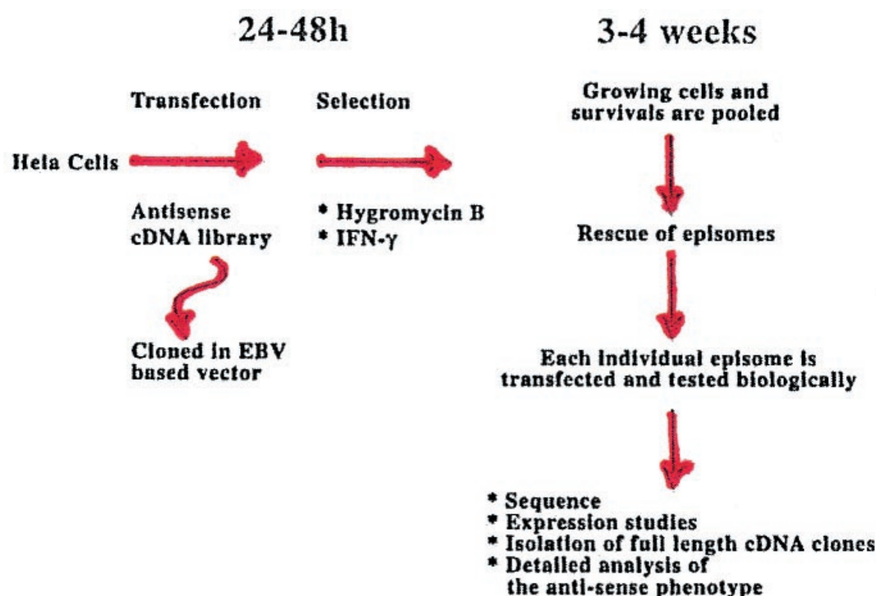

Figure 1 Outline of the TKO strategy, applied in IFN-γ-treated HeLa cells. The cDNA fragments that were scored as positives in a second round of transfection were subjected to further analysis, and are described in Table 1. (For more details see Deiss and Kimchi, 1991; Deiss *et al*., 1995)

cells) was introduced by transfection into HeLa cells. The entire cell population was then exposed for 3–4 weeks to IFN-γ and the hygromycin B selection drug. Finally, surviving cells were isolated, and episomal vectors rescued from these cells were individually tested in a second round of transfection/selection in HeLa cells, in order to confirm the phenotype. The plasmids that were scored as positive clones in the second round were further analysed. Southern and Northern blot analysis classified the functional antisense fragments carried by these vectors into seven non-overlapping groups of cDNAs, corresponding to seven different genes (Table 1). Sequence analysis indicated that five groups corresponded to novel genes. We named them: Death Associated Protein (DAP) genes, DAP-1 to DAP-5 (Table 1; DAP-2 is also called DAP-kinase). Two groups represented known genes: the thioredoxin gene, whose product is involved in redox regulation of intramolecular disulfide bonds in proteins; and cathepsin-D, which is an aspartyl lysosomal protease. Basic information concerning the size of mRNAs encoded by the selected genes, as well as their chromosomal assignment, is shown in Table 1.

From that point, we focused on the molecular and functional characterization of the novel DAP genes. Towards this aim, the full-length sense cDNAs were isolated, specific antibodies were raised against the recombinant proteins, and the expression, function, and biochemical properties of the proteins were studied. In addition, the possible link of one of these genes to cancer has been addressed, as well as the unpredicted finding that an aspartyl protease is essential for apoptosis.

Table 1 The rescued functional cDNA fragments

Rescued cDNA #	*Length of cDNA fragment* (bp)	*Size of mRNA* (Kb)	*Identity*	*Chromosomal assignment*
230, 254, 255, 264, 258	320	2.4	DAP-1	5p15.2
256	367	6.3	DAP-kinase	9q34
259	252	1.7	DAP-3	1q21
253	200	4.0	DAP-4	Unknown
260	763	3.8	DAP-5	11p15
229	370	2.2	cathespin D	11p15
241, 248, 251, 252	350	0.7	thioredoxin	9q31

DAP-kinase studies

The study of this gene provides an interesting paradigm, illustrating how from the initial selection of death-protective anti-sense cDNA fragment, we proceeded towards the structure/function analysis of the protein, and then to the notion that a central 'death gene', with a potential tumor suppressor activity, has been isolated.

Once the full-length cDNA clone of DAP-2 was isolated, the deduced amino acid structure predicted that a novel calmodulin-dependent serine/threonine type kinase (160 kDa) had been isolated (Figure 2). Its kinase domain has a classical 12 subdomain composition, typical of serine/threonine kinases, followed by a region that shares high homology with the calmodulin regulatory domains of other kinases. Adjacent to the latter, we found eight ankyrin repeats followed by two P-loop motifs (Deiss *et al*., 1995). This provided the first documented example of a serine/threonine kinase carrying ankyrin repeats, a motif involved in the formation of stable protein–protein complexes, which may mediate the interaction of DAP-kinase with its downstream or upstream effectors. Moreover, a typical death-domain module was identified at the 3′ end of the protein, followed by a stretch of amino acids that is rich in serines (Feinstein *et al*., 1995a). The death domain of DAP-kinase contains all the boxes of homology and the conserved amino acids characteristic of the analogous domains in other death domain-containing proteins (Feinstein *et al*., 1995a). Many of these other proteins have a direct involvement in programmed cell death, including the p55 TNF receptor, the Fas/APO-1 receptor, DR3-5, FADD/MORT-1, RIP, TRADD and RAIDD (Ashkenazi and Dixit, 1998). Being a motif composed of several α-helical regions, the death domain of DAP-kinase may prompt association with other protein partners critical for the function of this novel type of kinase. The serine rich C-terminal tail of DAP-kinase could negatively regulate a putative function of the death domain, as previously shown for the corresponding region in the Fas/APO-1 receptor (Feinstein *et al*., 1995a).

Altogether, the predictions made on the basis of the amino acid sequence suggested that a structurally unique kinase had been identified, and emphasized the importance of studying this novel death-associated protein both at the biochemical and functional levels.

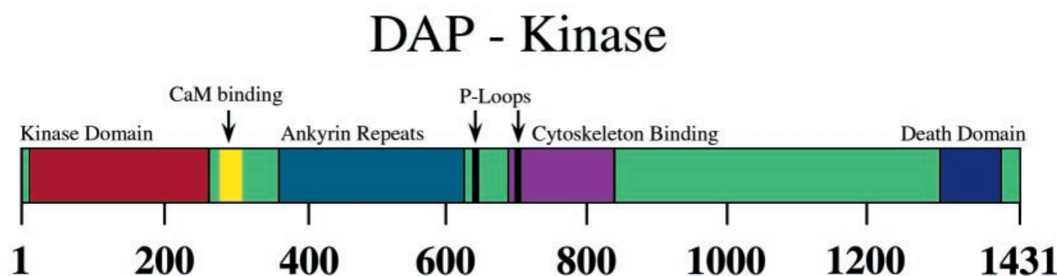

Figure 2 Schematic representation of DAP-kinase. The various motifs and domains as predicted by the deduced amino acid sequence and/or found by experimental work are shown. The numbers below indicate the amino acid positions

Biochemistry of DAP-kinase

A series of biochemical studies confirmed the structural predictions described above. The ability of the protein to phosphorylate itself and an exogenous substrate was shown by *in vitro* kinase assays. We proved that the recombinant protein was capable of directly binding calmodulin, and that the intrinsic kinase activity was stimulated by Ca^{2+}/calmodulin (Cohen *et al*., 1997). The removal of the calmodulin regulatory domain (ΔCaM mutant) generated a constitutively active kinase (i.e., a gain of function mutation). This is consistent with previous information on other well studied calmodulin-dependent kinases, in which the calmodulin-regulatory domain has inhibitory effects on the kinase activity, relieved by binding to Ca^{2+}/calmodulin (Shoemaker *et al*., 1990). DAP-kinase activity was abolished by the substitution of a conserved lysine by alanine residue within the kinase domain (K42A), thus generating an inactive kinase mutant with potential dominant-negative activity (Cohen *et al*., 1997). Altogether, the complex structure of DAP-kinase that carries multiple functional domains and motifs, places it at an important functional junction within the branched network that leads to cell death, where it may receive and release a variety of input and output signals.

The cellular function of DAP-kinase

The structural studies of DAP-kinase were then complemented by functional assays. We found that overexpression of wild type DAP-kinase reduced the number of viable clones in HeLa cells (Cohen *et al*., 1997). Detailed examination of the cells, early after the transfection (24–48 h), indicated that overexpression of DAP-kinase killed the cells. The constitutively active ΔCaM mutant had stronger death-inducing effects in these overexpression assays. In contrast, the catalytically inactive kinase (K42A) completely lost its death inducing activity, and instead, its introduction slightly increased the number and the size of stable clones. The induction of cell death therefore strictly depended on the status of the intrinsic kinase activity, and the catalytic activity of this protein was clearly required for the death-promoting function of this protein. These overexpression experiments provided a second independent functional support for DAP-kinase being a death gene, in addition to the anti-sense-RNA-mediated protection.

The above-mentioned observations concerning the subtle stimulatory effects that K42A DAP-kinase mutant exerted on the growth of transfected HeLa clones, promoted us to test more directly whether this catalytically inactive form of the kinase could function in a trans-dominant negative manner. We found that transfections with the catalytically inactive K42A mutant protected cells from the IFN-γ-induced cell death (Cohen *et al*., 1997). These studies closed a circle which started by the rescue of a death-protective anti-sense cDNA fragment, which interfered with protein expression, and ended by finding that a mutant form of the full-length DAP-kinase exerted similar effects, probably by interfering with the function of the endogenous protein.

Additional sets of experiments further strengthened the notion that DAP-kinase is a central cell death gene participating in several apoptotic systems. DAP-kinase gene was found to be widely expressed in many cells and tissues. In addition, it was found that DAP-kinase is involved in cell death induced by other cytokines, such as TNF-α, as well as death induced by detachment from extracellular matrix (Inbal *et al*., 1997). Since the early signaling cascades generated by p55 TNF receptor, or detachment from extracellular matrix do not share any known common elements with IFN-γ early signaling, DAP-kinase is likely to lie further downstream, and may function as a universal target at which different apoptotic signals eventually converge. This is consistent with the original design of the TKO approach, which was aimed at targeting genes that function downstream to the receptor-generated early signaling. The latter was achieved by introducing IFN-responsive DNA sequences in the expression cassette driving the cDNA library, a step which made the selection dependent on intact IFN early signaling, and thus increased the probability of isolating downstream components of the pathway (Deiss and Kimchi, 1991).

Another important issue relates to the mode of DAP-kinase regulation during programmed cell death. It was found that DAP-kinase mRNA levels were moderately increased in response to IFN-γ in HeLa cells (Deiss *et al*., 1995). Yet, we suspect that this may not be the major regulatory step. DAP-kinase protein may be regulated at the post-translational level, and the issue of DAP-kinase activation by a variety of apoptotic signals is currently under an extensive study.

Intracellular localization of DAP-kinase

Immunostaining indicated that DAP-kinase is localized to the cytoskeleton in association with the microfilament system. This was further supported by biochemical fractionations, which indicated that DAP-kinase remained insoluble upon mild detergent extractions, and by application of cytoskeletal disrupting drugs. Treatment of cells with nocodazole, which disrupts the microtubule system, did not affect the solubility of DAP-kinase. Conversely, latrunculin A, a drug which disrupts the actin-microfilament system, released a significant portion of DAP-kinase molecules into the detergent-soluble fraction (Cohen *et al*., 1997). Deletion analysis of DAP-kinase mapped the region mediating cytoskeleton binding to a stretch of 200 amino acids localized downstream to the first P-loop motif (Figure 2). Most interestingly, cell killing by overexpression of DAP-kinase depended on its correct localization to the cytoskeleton. A mutant of DAP-kinase which was mislocalized to the nucleus did not impose the typical changes in the actin-microfilament organization and cell death. This was an important finding since loss of stress fibres and disruption of microfilament organization occur at early stages of epithelial and fibroblastic cell death in response to different apoptotic stimuli. The specific intracellular localization of the kinase may therefore provide a mechanistic clue as to how external signals impose these cytoskeletal changes (Cohen *et al*., 1997).

Implications of DAP-kinase in tumorigenesis

Positive mediators of cell death may be lost or inactivated in tumors and therefore may function as tumor suppressor genes, as clearly documented in the case of p53. Chromosomal localization studies mapped DAP-kinase to human chromosome 9 band q34.1 (Feinstein *et al*., 1995b) – a region prone to translocations in human leukemias – and to LOH in bladder carcinomas. In an attempt to elucidate a role for DAP-kinase in oncogenesis, we first tested the expression of DAP-kinase in human cell lines derived from different neoplasms. DAP-kinase mRNA and protein expression levels were below detection limits in 70% of B-cell lymphoma and leukemia cell lines and in 30% of cell lines derived from bladder carcinomas, breast carcinomas, and renal cell carcinomas (Kissil *et al*., 1997). This stood in sharp contrast to the finding that DAP-kinase mRNA was widely expressed in all the tested human and murine tissues, as well as in many immortalized cell lines established from normal cells.

In two bladder carcinoma and in one of the B cell lymphoma cell lines, DAP-kinase expression could be restored by treatment of cells with 5-aza-2′-deoxycytidine, a drug which inhibits DNA methylation (Kissil *et al*., 1997). This suggested that loss of expression in these particular cases was due to DNA methylation, as previously reported for other tumor suppressor genes, such as p16, VHL and pRB (reviewed by Laird, 1997). Yet, we found, from the tumor cell lines screen, that demethylation is not an exclusive mechanism for suppressing DAP-kinase expression, in accordance with the well established paradigm that tumor suppressor genes may be lost or inactivated by multiple genetic or epigenetic alterations. These experiments provided the first hint that DAP-kinase inactivation may possibly be a causative factor in the formation of tumors, as was further tested in experimental animal model systems. Obviously, a major goal in our laboratory is to enter into a large-scale screen of normally occurring human cancers, to determine the frequency of genetic and epigenetic changes of DAP-kinase at different stages of tumor growth.

In parallel, we have carried out a second functional approach that directly tested in animal models, whether the DAP-kinase gene has tumor suppressor activity. In these experiments, we restored normal expression levels of DAP-kinase into tumor cells that have lost it, and assayed the impact of this genetic manipulation on the tumorigenic properties of these cells. This approach became even more attractive once we found that high-metastatic lung carcinoma clones, originating from two independent murine lung tumors, lacked DAP-kinase expression, in contrast to their low-metastatic counterparts which expressed normal levels (Inbal *et al*., 1997). FLAG-tagged wild-type DAP-kinase was introduced into the high-metastatic Lewis carcinoma cells. Stable transfected clones in which DAP-kinase expression was restored to physiological levels displayed a normal pattern of anchorage-dependent cell growth *in vitro*. These transfectants were therefore assayed for their tumorigenic and metastatic activity in syngeneic mice. Strikingly, restoration of physiological levels of DAP-kinase into the high-metastatic Lewis carcinoma cells suppressed their ability to form lung metastases after intravenous injections into mice. The *in vitro* effects were proportional to the levels of the ectopically expressed DAP-kinase, and even the low-expressors, containing levels which were below the normal levels, displayed reduced metastatic activity. The transgene also delayed local tumor growth in a foreign micro environment, yet this feature was less sensitive to DAP-kinase ectopic expression than the metastatic activity (Inbal *et al*., 1997).

By selecting *in vivo* rare lung metastases, after injections of the original low-metastatic cells into irradiated syngeneic mice, we found that the development of these metastatic lesions correlated with loss of the endogenous DAP-kinase expression. Moreover, treatment of cells recovered from one of these DAP-kinase negative lung lesions with 5-aza-2′-deoxycytidine, restored protein expression to the normal levels. DNA methylation was therefore also responsible for silencing the endogenous DAP-kinase gene in some of the *in vivo* selected lung lesions (Inbal *et al*., 1997). Altogether, these experiments suggested that loss of DAP-kinase expression provides a positive selective advantage during the formation of lung metastases.

Next, the mechanisms underlying the suppressive effects of DAP-kinase on metastasis and local tumor growth were studied. *In situ* TUNEL staining was performed on histological sections of local tumors. It was found that the apoptotic index in the slow growing local tumors, formed by the DAP-kinase-transfected cells, was higher than the value measured in the tumor mass formed by the control clone (Figure 3). These

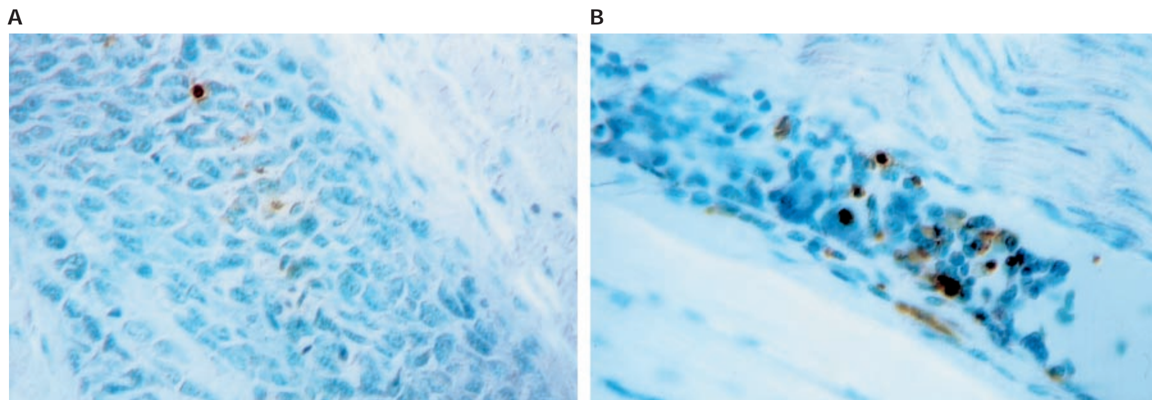

Figure 3 Exogenous expression of DAP-kinase increases the apoptotic index in the animal context. *In situ* TUNEL staining of tumor sections from Lewis lung carcinoma cells that are (**a**) null for DAP kinase expression; (**b**) transfected with DAP-kinase. The brown staining corresponds to apoptotic cells

results provided the first indication implicating the DAP-kinase gene in augmenting the threshold sensitivity of the tumor cells to apoptotic signals *in vivo*. To directly address this issue, the transfected cells were exposed *in vitro* to two types of apoptotic stimuli of the sort encountered by metastasizing cells at the different stages of malignancy, such as TNF-α and anchorage-independent cell growth. The transfectants displayed higher sensitivity to the apoptotic effects of TNF-α: fragmented nuclei appeared much faster, and total cell death was greater. A second type of an apoptotic stress was induced by growing cells under anchorage-independent conditions in soft agar. In contrast to the parental high-metastatic clone which formed large viable colonies in semi-solid medium, the various DAP-kinase transfected clones formed small colonies comprising of apoptotic cells. Based on these experiments it is suggested that the DAP-kinase-mediated suppression of metastasis results, at least in part from increased sensitivity to various death-inducing stimuli. It is proposed that loss of DAP-kinase expression provides a novel mechanism that links suppression of apoptosis to metastasis (Inbal *et al*., 1997).

DAP-3 studies

The DAP-3 gene, localized to chromosome 1 band q21 (Kissil and Kimchi, 1997), codes for a protein of 46 kDa which carries a potential 'P-loop' motif, suggesting that it is a nucleotide binding protein. The gene is transcribed into a single 1.7 kb mRNA, which is ubiquitously expressed in different cells and tissues (Kissil *et al*., 1995). The notion that DAP-3 functions as a major positive mediator of cell death was supported by two independent lines of evidence. One of them is based on the original assay – which in fact led to the identification of this gene, i.e., protection from cell death conferred by reduced expression of the protein. The assay in this experiment was based on neutral dye uptake into viable cells. It showed that transfection with a vector expressing anti-sense DAP-3 RNA caused a significant increase in the fraction of cells that remained viable in the continuous presence of IFN-γ. Western blot analysis, performed by using anti-DAP-3 polyclonal antibodies, confirmed that the anti-sense RNA expression indeed reduced the steady state levels of DAP-3 protein in the continuous presence of IFN-γ (Kissil *et al*., 1995).

The second line of evidence came from overexpression of the full-length sense cDNA. Conceptually, it is well accepted now that overexpression of a single gene that is part of a molecular pathway that leads to cell death, is often itself sufficient to trigger the entire cell death process, even in cases where the normal regulation of the gene during apoptosis does not involve elevation of expression. We found in this respect that ectopic expression of DAP-3 from a constitutive promoter induced the death of HeLa cells within 48 – 72 h, leading to a significant reduction in the number of drug resistant stable clones after 2 weeks (Kissil *et al*., 1995). Thus, the finding that overexpression of DAP-3 induced cell death provides an independent support for this gene, being a positive mediator of cell death. The DAP-3-induced cell death was considerably weakened by introducing a single amino acid mutation within the P-loop motif. This indicates that the nucleotide-binding property is critical for the death promoting functions of the protein (Kissil *et al*., 1999).

DAP-5 studies

DAP-5 gene codes for a 97 kDa protein, that is highly homologous to translation initiation factor 4G (eIF4GI; p220), with an overall 27% identity and 48% similarity between the two at the amino acid level (Levy-Strumpf *et al*., 1997). eIF4GI functions as a scaffold for assembling several initiation factors that direct ribosomes to RNA at the cap site. The N-terminal part of eIF4GI binds eIF4E, the well known cap-binding protein, whereas the middle region of the protein binds eIF3 and eIF4A translation initiation factors. The cap binding end of eIF4GI is cleaved during lytic infection with some RNA viruses by a cellular protease. By this mechanism the virus inhibits the cellular cap dependent translation and recruits the translation machinery exclusively towards the un-capped viral RNA. Strikingly, the DAP-5 protein lacks the N-terminal region that is necessary for cap-dependent translation while it shares high homology at the core region which binds to eIF3 and eIF4A. The C-terminus part of the protein shares less homology to eIF4GI. Thus, DAP-5 may represent a naturally occurring form of the cleaved eIF4GI, linking translational control to the process of cell death (Levy-Strumpf *et al*., 1997).

Interestingly, the DAP-5 cDNA fragment which was rescued by the TKO selection was a sense fragment. It coded for a mini-protein that encompassed the less conserved C-terminal part of the protein (see the scheme in Figure 4). The domain structure of DAP-5 predicts that this selected death-protecting mini protein, depleted of its eIF3 and eIF4A-binding sites, should function in a dominant-negative manner; the reduced homology to eIF4GI within this protein fragment predicts that it might compete specifically with the full-length DAP-5 (Levy-Strumpf *et al*., 1997). The two yeast eIF4G homologs lack the C-terminal region of the protein, suggesting that this region was acquired later in evolution and possibly carries a regulatory function. This regulatory region is targeted by the sense cDNA fragment that was isolated.

What is the biochemical function of DAP-5? One angle in this issue was studied by the group of N Sonenberg who independently isolated this gene and named it p97. This group has shown that DAP-5/p97 protein binds to eIF3 and eIF4A, and that like the cleaved eIF4GI, it fails to bind the cap-binding protein eIF4E (Imataka *et al*., 1997). Assays based on transient transfections showed that DAP-5/p97 overexpression repressed both cap-dependent translation as well as cap-independent translation from EMCV internal ribosomal entry sites (IRES). In contrast, eIF4G supported both translation pathways. Based on these transfection studies, they suggested that DAP-5/p97 may function as a repressor of translation that titrates out eIF3 and eIF4A from cells. Later Imataka and Sonenberg (1997) reported that unlike eIF4GI which possesses two independent eIF4A-binding sites (one at the central core, and the other in the C-terminus),

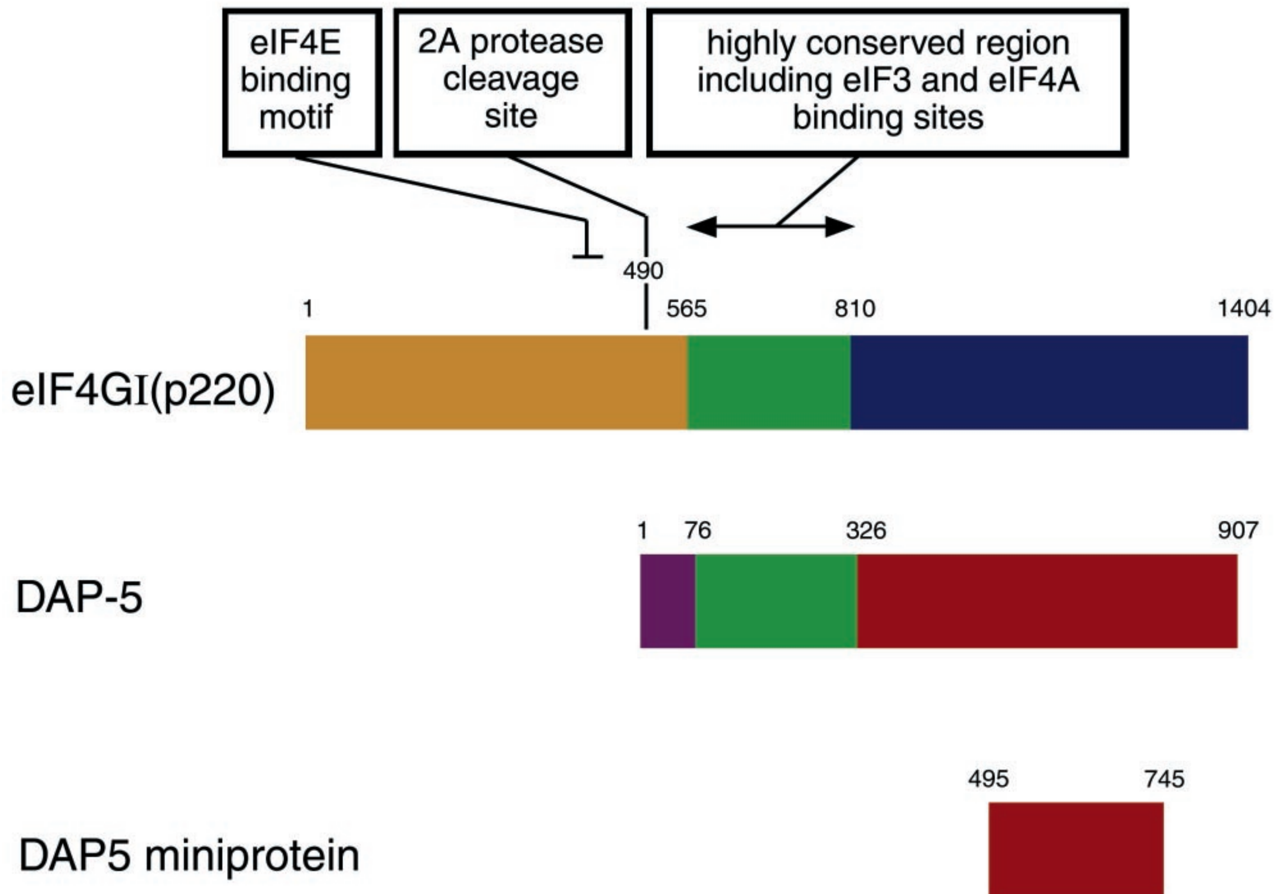

Figure 4 Schematic representation of DAP-5 protein aligned to eIF4GI translation initiation factor. The most conserved region of homology is marked by a common color. The position of the rescued death-protective mini-protein is shown. The numbers indicate the amino acid positions

DAP-5 possesses a single binding site. According to their interpretation, the lack of the second binding site could prevent a possible function of DAP-5/p97 as a cap-independent translational factor, thus supporting the repressor hypothesis mentioned above. However another recently cloned protein belonging to the eIF4G family, PAIP, enhanced translation in the absence of the second C-terminal eIF4A-binding site (Craig *et al*., 1998). Another finding which is not compatible with the simple repressor hypothesis, is the constitutive and abundant expression of DAP-5/p97 in normally growing cells. This raised an alternative working model which proposed that during apoptosis or other stress conditions, DAP-5/p97 may be modified, by specific transcription/translation regulatory processes, into novel forms which fulfill the death-promoting functions of this gene. Indeed, in a recent series of experiments we found that during cell death, initiated by Fas receptors or by p53 activation, p97/DAP-5 was rapidly converted into a novel truncated form, lacking the C-terminus. It is possible that this novel form of the protein may drive cap-independent translation of certain critical cellular mRNAs required for each cell death, an open issue which awaits further investigation.

Another line of research recently ended up, quite surprisingly, in the independent cloning of DAP-5 gene. In this work Yamanaka *et al*. (1997), developed an elegant strategy to clone novel targets for the RNA editing enzyme, apobec-1. Apobec-1 is the catalytic subunit of the editosome multi-protein complex which substitutes cytidine to uridine by deaminase activity. The strategy was based on a modified differential display technique to selectively amplify mRNAs that were edited in the livers of transgenic mice over-expressing apobec-1. By doing so, they identified a novel target which they called NAT1 (for novel apobec-1 target no. 1). NAT1 mRNA was extensively edited at multiple sites in these livers, creating many stop codons. It should be mentioned that although this artificial system of hyperediting fails to provide the precise physiological editing sites along the RNA molecule, it definitively scores the real potential targets for the apobec-1 activity. Surprisingly, NAT1 was identified as the mouse homolog of the human DAP-5 gene (Yamanaka *et al*., 1997). This work opens up the possibility that apobec-1-mediated DAP-5 RNA editing might be one of a few mechanisms which regulate DAP-5 function by generating novel forms of the protein.

DAP-1 studies

DAP-1 is a 15 kDa, proline rich, basic protein. It has two potential cdk phosphorylation sites; the phosphorylated DAP-1 protein displayed a different pattern of mobility on gels (Deiss *et al*., 1995). Immunostaining and biochemical fractionation established that this

protein is localized in the cytoplasm. Yet, no clues were so far provided concerning the biochemical function of this protein. At the cellular level, the death-promoting effects of the protein were prominent, and overexpression of the full-length protein potentiated the killing effects of IFN-γ (T Raveh and A Kimchi, unpublished results).

The cathepsin D protease connection to apoptosis

One of the surprises in the TKO selection was the identification of the insert carried by plasmid #229 as an anti-sense cDNA fragment directed against human cathepsin D aspartic protease (Table 1). Obviously, proteases were expected to be isolated during the selection, yet our prediction was that they will fall into the known family of caspases. The lysosomal cathepsin D has a wide range of activities in mammalian cells, mostly executed in the lysosomal compartment during steady state growth conditions. It was only after a detailed study of the fate of this protease during cell death, that an interesting unexpected scenario has emerged, consistent with an active role in apoptosis (Deiss *et al*., 1996).

The initial analysis of the secondary stable clones indicated that the high levels of cathepsin D anti-sense RNA protected the HeLa cells from IFN-γ and Fas/APO-1-induced death. Since pepstatin A, a peptide inhibitor of aspartic proteases, was extensively used in the past to specifically suppress cathepsin D activity in intact cells, we used it in our systems. It was found that this peptide inhibitor suppressed cell death in HeLa cells exposed to IFN-γ and to the anti-Fas/APO-1 agonistic antibodies. Protection by pepstatin A was also detected during the TNF-α-induced programmed cell death of U937 cells (Deiss *et al*., 1996). We thus established the role of this protease in three different apoptotic systems.

The next challenge was to understand how a housekeeping lysosomal protease is recruited to the tightly regulated process of programmed cell death. We found that this gene has an interesting pattern of regulation during cell death. The mRNA expression of cathepsin D was elevated and the processing of the protein was regulated in the dying cells. As a consequence, high steady-state levels of an intermediate, proteolytically-active, single chain form of this protease accumulated in cells (Deiss *et al*., 1996). This form is present at low steady-state levels in normally growing cells, and is usually found in pre-lysosomal compartments. Its abnormal accumulation in apoptotic cells further suggested that changes in cathepsin D intracellular localization, and hence in the spectrum of potential substrates might occur during cell death.

Recently, El-Deiry and co-workers reported that cathepsin D has a role in p53-dependent apoptosis. Using subtractive hybridization screening, they isolated cathepsin D as an upregulated transcript appearing in cells undergoing adriamycin-induced apoptosis, in a p53-dependent manner (Wu *et al*., 1998). They found that cathepsin D mRNA levels increased in wild-type p53-expressing cell lines following adriamycin addition. Other DNA damaging reagents were similarly effective in elevating cathepsin D expression. Two p53 DNA-binding sites were identified in the cathepsin D promoter. They bound to p53 *in vitro*, and appeared to mediate transactivation by p53 of a reporter gene driven by the cathepsin D promoter. It appears from this work that cathepsin D also mediates apoptotic signals that involve DNA damage and are p53-dependent, thus further extending the spectrum of apoptotic signals that use this aspartic protease to intracellular signals as well.

Altogether, cathepsin D may be added to the growing list of proteases that function as positive mediators of apoptosis. How its function is related to the cascade of caspase activation during apoptosis is an issue to be studied.

Conclusions and prospects for the future

While the research on the novel DAP genes is still very far from completion, a few common interesting paradigms already emerge. The start points in the isolation of these genes were the selected short cDNA fragments, which in most cases directed the transcription of high levels of anti-sense RNAs. In the case of DAP-5, a mini protein was translated from the functional sense cDNA fragment. This means that the selection worked by either reducing the levels or suppressing the function/activation of the corresponding proteins. Later on, once the full-length proteins were further characterized, we designed specific mutations within the DAP genes, or used small peptide inhibitors, to illustrate by a few other independent approaches the rate limiting functions that these genes display in apoptosis. Two classical examples discussed in this paper are the K42A dominant negative mutant of DAP-kinase, and the pepstatin A peptide inhibitor, which suppressed the function of DAP-kinase and of cathepsin D proteins, respectively. These reagents displayed cell death protective effects similar in their nature to the effects of the original anti-sense cDNAs, thus confirming by a few independent molecular tools that these proteins are indispensable in some of the apoptotic associated events.

It should be emphasized that genes identified by virtue of their rate limiting function in apoptosis, as performed in these studies, could either interact indirectly with the main death pathways, or be central components of the main death pathways themselves. A few criteria should distinguish between the two possibilities; the detailed study of these criteria indicate very clearly that some of the DAP genes are integral components of death pathways.

One of the most important criterion relates to the issue of regulation. If the studies indicate that a gene, which was initially rescued through its function, is actually tightly regulated by the apoptotic trigger, the probability increases that an intrinsic component of the apoptotic pathway has been selected. The regulation may take place at multiple different levels, including transcriptional, post-transcriptional, translational and post-translational. One of the examples discussed in this paper, is the cathepsin D. Following its rescue as a gene whose function is rate limiting during apoptosis, it was found that the gene is tightly regulated at a few independent levels. First, it was found that different killing cytokines elevated the cathepsin D mRNA expression. Second, a specific alteration in the

processing of this protein occurred during apoptosis, resulting in the accumulation of an active prelysosomal single chain precursor. And third, p53 binding sites were identified in the promoter region, elevating the transcription of the gene in response to DNA damage. Also, the regulation of DAP-kinase and of DAP-5 by the different apoptotic triggers, is under extensive study in our laboratory, as detailed above.

The second criterion refers to the range of apoptotic triggers which depend on the rescued gene. If DAP gene's function is not restricted to a single external apoptotic stimulus, then a more general role in death pathway(s) may be attributed to the gene. For example: DAP-kinase, and cathepsin D mediate Fas and TNF-α-induced cell death in addition to mediating the IFN-γ effects. Moreover, DAP-kinase also mediates cell death induced by detachment from extracellular matrix (named anoikis). Altogether, the spectrum of apoptotic stimuli that converge into DAP-genes seems to be very wide, suggesting that they serve as common downstream mediators to various signals.

DAP-kinase, DAP-3, DAP-1, and DAP-5 were highly conserved in evolution. Recently we have isolated the *C. elegans* homolog of DAP-3, which displays 35% identity at the amino acid level (Kissil *et al*., 1999). Moreover, each of these genes represents the prototype of a novel gene family. In this respect, two novel DAP-kinase homologs which share 80% identity in their catalytic domains, were recently identified. No homology was found outside of the catalytic domain. One of the homologs, named ZIP-kinase, contains leucine zipper structure at its C terminus and was localized to the nuclei of cells (Kawal *et al*., 1998). It displays apoptotic functions which like in the case of DAP-kinase depend on its intrinsic kinase activity. Its rat counterpart was also independently cloned and characterized by Kogel *et al*. (1998). DAP-5 belongs to the extended eIF4G family consisting of the aforementioned eIFGI, and the recently cloned eIF4GII (Gradi *et al*., 1998), and PAIP (Craig *et al*., 1998). A family of DAP-1 related proteins was recently identified by the EST data bank searches.

The ubiquitous expression of these genes in many cell types and tissues, and the high degree of conservation among different organisms imply that we managed to isolate evolutionary conserved and important components of cell death pathways that are common to various cells.

Finally, the link to cancer and to other pathologies is another important criterion in evaluating the physiological and pivotal role of DAP genes. The best indications that we have so far relate to the DAP-kinase gene which possesses strong anti-metastatic activities. This aspect in the function of the gene was attributed at least partially, to the high susceptibility that it conferred to apoptotic stimuli, some of which operate during metastasis. For instance, upon entering into circulation, the invading metastasizing cells may be killed by interactions with monocytes, natural killer cells, and neutrophils, by exposure to cytokines, to nitric oxide anions, or by different mechanical stresses. The loss of DAP-kinase conveys to the metastasizing cells strong positive selection to resist the apoptotic stress, as also shown by the *in vivo* selections of lung metastases in mice model systems. Frequent inactivating mutations in human tumors, if detected, should provide an additional independent support that DAP genes display a major function in keeping cells under the tight control of apoptotic mechanisms that take place normally *in vivo*. Obviously, other mechanisms may contribute to the tumor suppressive functions of DAP genes, in addition to mediating apoptotic responses.

Intriguingly, the genes which were rescued code for proteins that have a diverse spectrum of biochemical activities, suggesting that we managed to hit multiple elements along cell death pathway(s). These activities include a structurally unique calcium/calmodulin-dependent kinase (DAP-kinase), a nucleotide binding protein (DAP-3), an aspartyl lysosomal protease (cathepsin D), a novel translation initiation factor (DAP-5), and a small proline-rich cytoplasmic protein (DAP-1). The major challenge now is to analyse how these diverse activities constitute biochemical pathway(s) leading to programmed cell death. This depends on studying the nature of interactions between the individual DAP genes on the one hand, and determining their functional position with respect to the other well known positive and negative mediators of apoptosis, on the other hand. For instance, the functional interaction between the different DAP genes can be worked out by trying to rescue the death-promoting effects of one DAP gene by the dominant negative mutant of another DAP gene, or by studying combinatorial death-promoting effects by double and triple transfections. The possible functional interactions with members of the bcl-2 family, or with the caspases, can be assessed now in a similar manner, by stable or transient transfections, or by the use of specific peptide inhibitors. Caspase could function in parallel to the biochemical pathways which involve DAP genes, or alternatively may be integrated into these pathways either as upstream modulators, and/or downstream executors.

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