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# DAP genes: novel apoptotic genes isolated by a functional approach to gene cloning

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## 1. Introduction

Cell cycle arrest, programmed cell death, and cellular senescence are three distinct processes that form part of the growth-restrictive scenarios that counteract cell proliferation. Among these processes, the study of programmed cell death (apoptosis) has gained significant momentum during the past few years. Programmed cell death is a genetically controlled response for 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 to cells 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: [1–6]). In addition, 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) [7].

Several types of programmed cell death have been characterized according to different morphological characteristics. Altogether, 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, generation of apoptotic

bodies that are rapidly phagocytosed by neighboring cells [8–10]. 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 molecular mechanisms of programmed cell death, emerging from studying both lower invertebrates and mammalian systems (see for example, Refs. [11–15]). Yet, it is clear that enormous gaps still exist in our knowledge which means that novel approaches and different insights should be introduced into this field. This review 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.

## 2. Genetic and molecular approaches so far used for isolating cell death genes

There is no doubt today that the powerful genetic tools available in invertebrate model organisms (i.e., collections of mutations that affect cell death, followed by the rescue of the relevant genes) have provided the most elegant approach for isolating cell death promoting, as well as cell death suppressing genes. The first important clues came from genetic studies of the nematode *Caenorhabditis elegans* (reviewed in Ref. [16]). They provided the initial information on the evolutionarily conserved families of genes that lie at the central core of the cell death program. In the nematode, this core consists of two genes, *ced-4* and *ced-3*, which function as positive mediators of programmed cell death [17–19], as well as a third gene, *ced-9*, which suppresses the action of *ced-4* and *ced-3* in cells that survive [20]. Vertebrate

homologues of *ced-3* have been then identified and studied in detail. They encode a family of intracellular cysteine proteases, called caspases (at least 11 members), which function as regulators/executors of cell death (for reviews see, Refs. [21–23]). Obviously, the identification of substrates to caspases, critical for mammalian cell death, is at the center of ongoing extensive research [24].

Another breakthrough discovery was the finding that the mammalian *bcl-2* gene shares functional and structural properties with the nematode *ced-9* suppressor of cell death [25]. *bcl-2* was first identified as an oncogene, involved in a typical chromosomal translocation in human B cell lymphoma which led to overproduction of the protein [26]. The functional relatedness to *ced-9*, and extensive work showing that it promoted the survival of many types of cells, provided the first milestone in a very important concept linking the suppression of cell death to oncogenesis. A number of Bcl-2-related proteins were later identified in mammals, and were extensively investigated over the past years [27]. Some, such as Bcl-2 and Bcl-XL inhibit programmed cell death, whereas others, such as Bax and Bak, promote programmed cell death. The basis for this dual function lies in the finding that the various Bcl-2 family members can dimerize, thereby antagonizing or enhancing each other's function.

As for *ced-4*, only recently a putative human homologue (named Apaf-1) has been cloned [28]. The isolation and identification of Apaf-1 as a potential *ced-4* homologue, and attempts to decipher the basis of the molecular interactions with the *bcl-2* suppressors of cell death and with the caspases, were prompted by different approaches. On the one hand, the genetic hierarchy in *C. elegans* placed *ced-4* upstream of *ced-3* [29,30], and on the other hand biochemical and molecular work, performed in mammalian systems, provided the initial functional clues. The molecular studies established the existence of direct physical interactions between CED-9/Bcl-XL and CED-4, which itself binds directly to some caspases [31–35]. The biochemical work proved a role for CED-4/Apaf-1 in caspase activation in cell free systems [28,36]. Moreover, cytochrome C which is released from mitochondria during cell death, in a manner which is suppressed by Bcl-2, was also implicated in the process of Ced-4/Apaf-1 activation.

This suggests altogether that at least two separate mechanisms may mediate the cross talk between these three evolutionary conserved elements of the central core of apoptosis [37].

*Drosophila melanogaster* is a second important invertebrate model that has been employed in a powerful genetic screen to isolate cell death genes. Three novel genes, reaper, hid, and grim, have been identified, that appear to be required for cell death during embryogenesis (reviewed in Ref. [38]). The mammalian homologues of reaper, hid and grim still wait to be identified (a weak homology to the death domain of the TNF receptor family was identified for reaper). These novel cell death genes function in parallel in activating the conserved caspase pathway. Together with their modifiers that will be isolated from second generation screens, they will extend very significantly our knowledge on apoptotic genes.

In mammals, one of the exciting approaches for studying the molecular basis of apoptosis was based on the finding that the genetic program of cell death can be initiated by activation of various well defined cell surface receptors. Among the external triggers which interact with such membranal receptors, cytokines belonging to the TNF family such as TNF- $\alpha$ , Fas/APO-1 ligand, and TRAIL [39–43], as well as members of other cytokine families such as interferon- $\gamma$  (IFN- $\gamma$ ) [44–48], and transforming growth factor- $\beta$  (TGF- $\beta$ ) [49], have been identified. These findings further suggested that exposure of mammalian cell cultures to a killing cytokine may provide a well controlled in vitro system for the isolation of genes that mediate programmed cell death. Some of the corresponding receptors like p55 TNF receptor-1, Fas/APO-1, DR3, DR4, and DR5 contain a critical intracellular domain, called death domain, necessary for receptor oligomerization and for recruiting specific intracellular proteins [50]. Yet, other cytokine receptors use different well characterized modes of signal transduction, such as the JAK/STAT pathway characteristic of IFN receptors [51], suggesting that various membrane-generated signals may finally converge into common downstream effectors/executors of programmed cell death.

Two main research directions exploited the great potential that resides in the cytokine-induced cell death systems. One study employed the yeast two-hybrid selection system for the rescue of adapter pro-

teins that directly interact with the death domain of p55 TNF receptor 1 and the Fas/APO-1 receptor, or with effector domains characterized in the novel adapter proteins. This led to the isolation of many novel molecules such as FADD/MORT-1, RIP, TRADD, FLICE/MACH-1 and DAXX [52–58] which function at the receptor proximal level of death domain-containing receptors, and transduce the apoptotic signals. The identification of FLICE/MACH-1 and of FLICE-2 as members of the caspase gene family (caspase 8 and caspase 10, respectively), provided a direct molecular link, not exclusive though, to the aforementioned central core of the cell death program.

The second direction, to which this review article is devoted, was the development of novel genetic screens in mammalian systems, for ‘hunting’ functionally relevant genes that constitute the death pathways. The general concept was to apply functional strategies of gene cloning in mammalian cells that undergo cytokine-induced programmed cell death, to identify novel cell death-promoting genes. We pioneered, several years ago, this specific direction of functional gene cloning [59], and succeeded in rescuing several novel positive mediators of cell death [48,59–62]. This review will first describe the critical steps of our technology (named TKO, for Technical Knock Out), pinpointing the important considerations and precautions which made this approach feasible. This will be followed by the characterization of several novel cell death genes which we managed to isolate by the TKO methodology, and by the analysis of their contribution to our current understanding of programmed cell death. Some other versions of functional gene cloning, which are based on the same concept of positive growth selection of mammalian cells, have recently led to the discovery of additional intriguing novel genes, as will be briefly described at the end.

Finally, it should be mentioned that in light of the tight linkage between loss of apoptotic responses and cancer development, a theme that immediately arises is whether some of the rescued novel apoptotic genes may turn out to possess tumor suppressive activity. Therefore, a special emphasis will be placed, in this review, on the role that functional approaches of gene cloning have played, and may continue to play, in the

isolation of novel candidates for tumor suppressor genes.

### **3. Development of the technical knock out (TKO) method: a functional approach to gene cloning**

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 *D. 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. 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 cytokine. 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 was chosen as the preferred external trigger mainly because over the years, we have characterized in detail the types of growth restrictive scenarios that this cytokine imposes on cells. We have reported in the past that interferons (IFNs,  $\alpha$ ,  $\beta$  and  $\gamma$ ) can either induce a reversible block in cell cycle progression or an irreversible apoptotic form of cell death, depending upon the nature of the target cells [48,63–66]. In some cell systems, we found that IFNs imposed a biphasic pattern of responses, i.e., proliferation arrest followed by programmed cell death, thus providing a unique system to study, within a single genetic constellation, the points where the cell cycle inhibitory and apoptotic mechanisms diverge [48]. Moreover, much information was available, at the time we started this approach, on the early signaling of IFNs. The latter included the discovery of specific cis-acting enhancer elements found

in a group of IFN-responsive genes (reviewed in Ref. [51]). Thus, we used this IFN-stimulated response element to increase the efficiency of expression from the anti-sense cDNA library during the selection. After extensive screening of many cell systems, various selection protocols, and different cloning strategies, we developed a feasible genetic screen, set to isolate positive mediators from interferon- $\gamma$  (IFN- $\gamma$ )-induced HeLa cells.

To make the TKO approach feasible, several conditions had to be fulfilled. First, high transfection efficiency was required, for allowing the representation of the entire cDNA library in a single round of transfection. Second, high levels of the anti-sense RNA transcripts were required during the selection process, to significantly reduce the corresponding protein levels. Third, the functional cDNA-carrying vector had to be easily rescued from the transfectants that have become IFN resistant, to enable the performance of a second cycle of selections. All these features resided in an EBV-based episomal vector that was constructed for this purpose (named pTKO1; [59]). The episomal nature of the vector provided multiple copies per cell, high frequency of stable transfectants, and simple plasmid rescue procedures. In addition, we introduced an IFN-stimulated response element into the expression cassette of the vector [59]. The latter step further elevated the anti-sense RNA expression in the IFN-selected cells, and thus more efficiently suppressed the corresponding protein expression during selection [48,60,61]. In retrospect, we estimate that this step was indeed critical for the success of this method. Moreover, since the activation of this enhancer depended on a functional JAK/STAT pathway, we decreased the probability of targeting upstream elements along the IFN early signaling cascades (e.g., IFN receptors, JAK kinases, STATs transcription factors) and increased the chances of cloning downstream central mediators of cell death.

#### 4. The rescue of functional cDNA fragments that protect cells from IFN- $\gamma$ -induced programmed cell death

A scheme delineating the major steps of the strategy is shown in Fig. 1. The cDNA library was

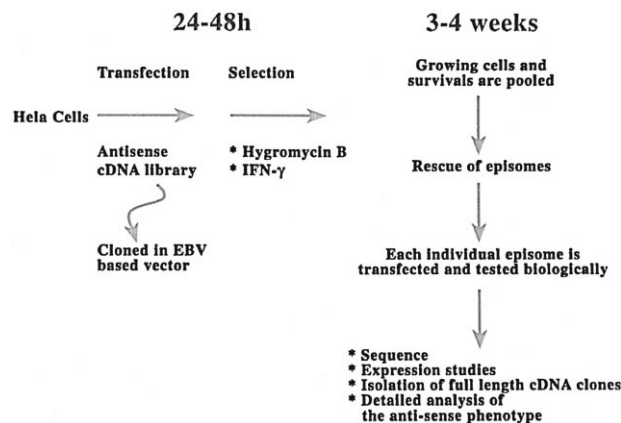


Fig. 1. Outline of the TKO strategy, applied in IFN- $\gamma$ -treated HeLa cells. The cDNA fragments that were scored as positives in a second round of transfection were subjected to further analysis, and are described in Table 1. For more details see Refs. [48,59].

prepared from RNA which was derived from a mixture of non-treated and IFN- $\gamma$ -treated HeLa cells (to include both constitutive and inducible mRNAs) and was cloned into the pTKO1 EBV-based vector in the anti-sense orientation. The anti-sense cDNA library was introduced by transfection into HeLa cells, and the cells were subjected to a double selection with IFN- $\gamma$  and hygromycin B. The stable transfectants that survived after long-term selection with the cytokine were pooled, and the episomal DNA was extracted. Each individual plasmid was then introduced into cells, in a second cycle of transfection, in order to test whether it was able by itself to reduce growth sensitivity to IFN- $\gamma$ . The plasmids that were scored as positive clones in the second round were further analyzed.

The first anti-sense cDNA clone isolated by this procedure, which rendered cells less sensitive to IFN- $\gamma$ , corresponded to the thioredoxin gene [59]. This proved that the cloning selection strategy is feasible and that redox regulation of proteins is an important step in the growth-restrictive effects of this cytokine. As a result of this initial success, we subsequently extended the rescue procedure and repeated it under more stringent conditions (i.e., higher IFN- $\gamma$  concentrations), in an attempt to rescue additional genes. Fourteen cDNA fragments, rescued by this large scale selection, were scored positive in the second round of confirmatory transfections. They were classified into seven non-overlapping groups of cDNAs, comprising multiple or single members, and corre-

Table 1  
The rescued functional cDNA fragments

Rescued cDNA #	Length of cDNA fragment (bp)	Size of mRNA (kb)	Identity	Resistant phenotype group
230, 254, 255, 264, 258	320	2.4	DAP-1	I
256	367	6.3	DAP-kinase	I
259	252	1.7	DAP-3	I
253	200	4.0	DAP-4	II
260	763	3.8	DAP-5	II
229	370	2.2	cathepsin D	I
241, 248, 251, 252	350	0.7	thioredoxin	II

sponding to seven different genes (Table 1). Sequence analysis indicated that nine out of the 14 cDNA clones were derived from five novel genes [48,60,62]. We named them: Death Associated Protein (DAP) genes, DAP-1 to DAP-5 (Table 1; DAP-2 is named DAP-kinase). Four cDNAs were identified as fragments of thioredoxin cDNA; another fragment (plasmid #229 in Table 1), was identical to human cathepsin D protease [61].

Each group recognized a single distinct mRNA on Northern blot (Table 1). The size of the anti-sense cDNA fragments, carried by these rescued plasmids ranged between 200–370 bp (with the exception of fragment #260 which was 763 bp long and turned out to be oriented in the vector at the sense direction; see below). As the work further progressed, we found that the small cDNA fragments were derived from different parts of the corresponding full length RNA transcripts with no common preferential position. Once antibodies were generated against the proteins, it was found that the anti-sense RNA expression from individual plasmids (i.e., anti-sense against DAP-1, DAP-kinase, DAP-3, and cathepsin D) reduced the steady state levels of the corresponding proteins during the IFN selection [48,60,61].

Detailed analysis of the transfected HeLa cells revealed a few interesting observations. In general, the response of HeLa cells to IFN- $\gamma$  consists of two phases. First, the cells stop proliferating, but still remain viable. This is then followed by massive cell death, characterized by reduction in cell size, cytoskeletal disorganization, rounding up and detachment from the plates, and nuclear chromatin condensation followed by its segmentation [48,67]. Transfection of each of the 14 plasmids into HeLa cells interfered with the cytokine-induced cell death, whereas cyto-

static responses appeared non-interrupted. Also the early signaling of IFN- $\gamma$  was unaffected. The ability of these cDNA fragments to exclusively protect cells from programmed cell death, without affecting growth arrest, provided the first indication that the genes that were rescued are positive mediators of cell death. Characterization of the IFN- $\gamma$  resistance phenotype which was generated by each of the rescued cDNAs, indicated that they could be divided into two major subgroups (Table 1). An example for type I resistance, conferred by the cathepsin D anti-sense cDNA fragment, is shown in Fig. 2. The assay in this

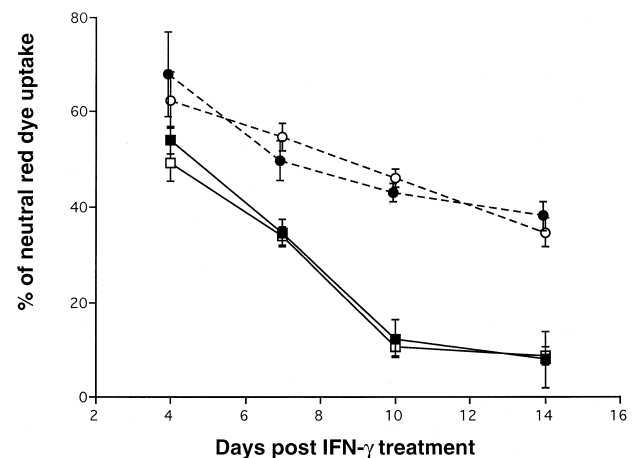


Fig. 2. Pattern of protection from IFN- $\gamma$ -induced cell death (type I resistance) conveyed by anti-cathepsin D RNA expression. A comparison between two control polyclonal populations of HeLa cells transfected with pTKO1-DHFR control vector (open and filled squares) and a pair of anti-cathepsin D-transfected polyclonal populations (transfected with pTKO1-229) (open and filled circles). The cells were treated with IFN- $\gamma$  (1000 U/ml), or left untreated. The fraction of viable cells was determined by comparing neutral dye uptake of IFN- $\gamma$ -treated cells to non-treated cultures at the indicated time points. Each point represents the average of quadruplicate determinations.

experiment is based on neutral dye uptake into viable cells and shows a significant increase in the fraction of transfected cells that remained viable in the continuous presence of the cytokine. Type II resistance phenotype was weaker than type I; the mechanisms responsible for this difference were later further investigated as detailed below and in Ref. [62]. It should be emphasized that a single cDNA fragment never conferred full resistance to IFN- $\gamma$ , as expected for genetic elements that function at distance from the receptor proximal level.

The focus of our research thereafter has been the detailed characterization of four of the novel DAP genes (DAP-1, -2, -3 and -5). The full length sense cDNAs were isolated, antibodies were raised, and the expression, function, and biochemical properties of these four proteins was studied. It turned out that these DAP proteins display various distinct biochemical features, reflecting intriguing mechanistic steps in apoptosis. The work on these four DAP genes will be described in this review, while no information will be provided on DAP-4 which is currently under study. In parallel, we investigated the unexpected involvement of cathepsin D protease in programmed cell death. We found that this aspartyl protease displays an interesting pattern of regulation during cell death, and that its proteolytic function is necessary for the execution of apoptosis (see below).

## 5. Characterization of the selected DAP genes

### 5.1. DAP-kinase

The study of this gene provides an interesting paradigm illustrating how from the initial functional selection of an anti-sense cDNA fragment, we pro-

ceeded 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. The description of DAP-kinase is therefore presented in more detail than that of the other rescued genes.

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 (see Fig. 3). Its kinase domain has a classical 11 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 [48]. 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 [50]. 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. 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 and TRADD [40,52–55,68,69]. Being a motif composed of several  $\alpha$ -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 do-

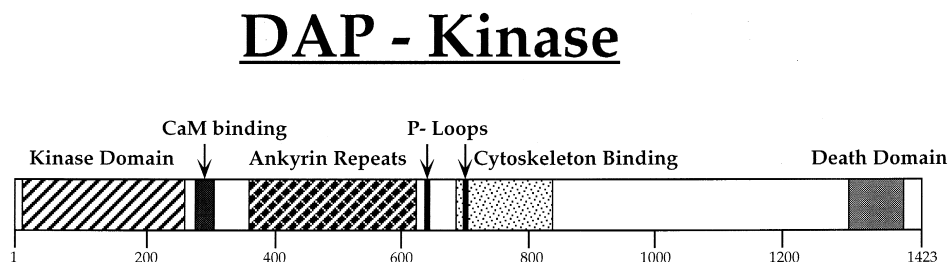
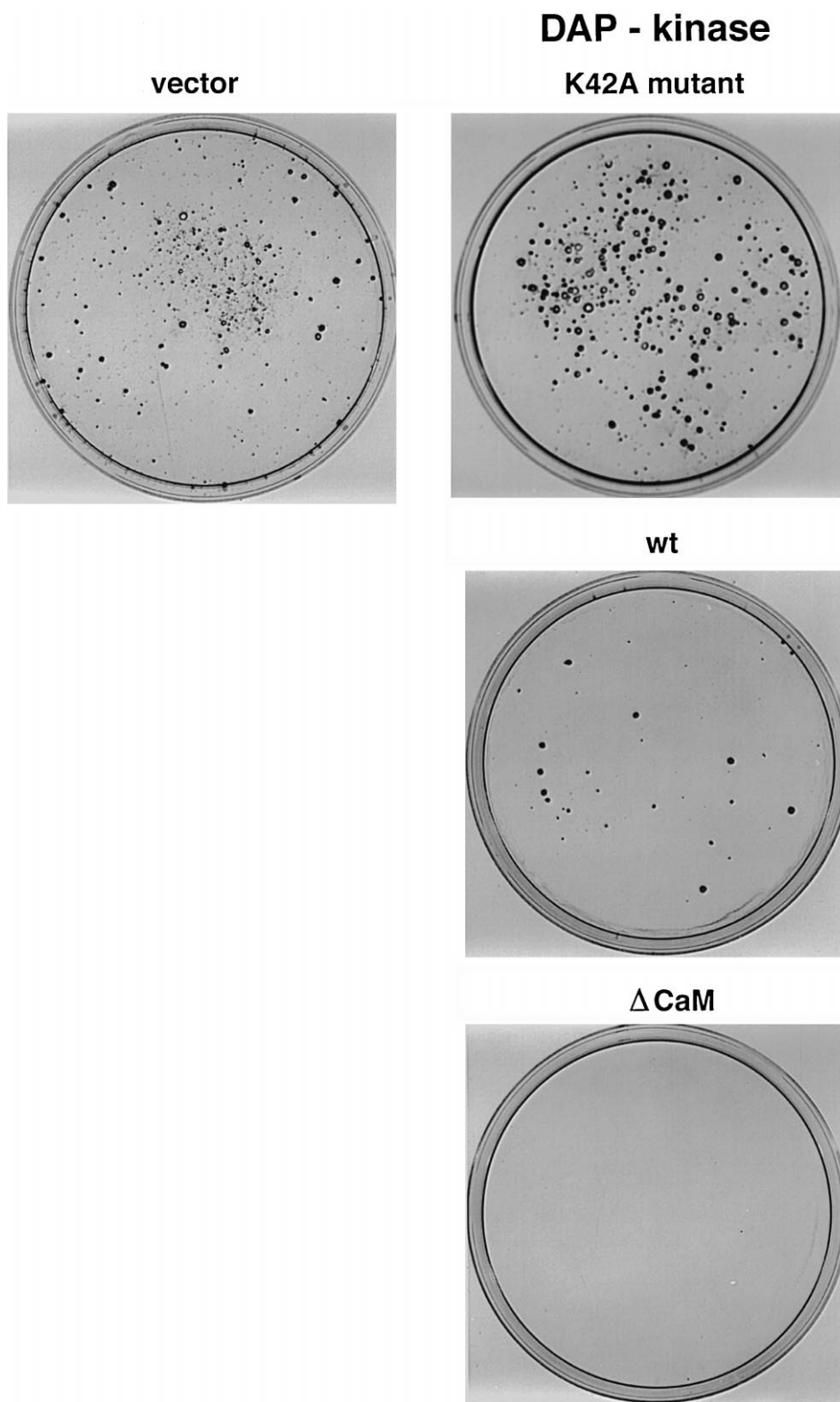


Fig. 3. Schematic representation of DAP-kinase. The various motifs and domains, as predicted by the deduced amino acid sequence and/or experimental work are shown. The numbers below indicate the amino acid positions.



main, as previously shown for the corresponding region in the Fas/APO-1 receptor [68].

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.

#### 5.1.1. 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  $\text{Ca}^{2+}$ /calmodulin [67]. The removal of the calmodulin regulatory domain ( $\Delta\text{CaM}$  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  $\text{Ca}^{2+}$ /calmodulin [70]. 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. Altogether, the complex structure of DAP-kinase that carries multiple functional domains and motifs, place 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.

#### 5.1.2. 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 [67]. Detailed examination of the cells, early after the transfection (24–48 h), indicated that overexpression of DAP-kinase killed the cells. The constitutively active  $\Delta\text{CaM}$  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 (Fig. 4). 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 abovementioned observations concerning the subtle stimulatory effects that K42A DAP-kinase mutant exerted on the growth of transfected HeLa clones (Fig. 4) prompted us to test more directly whether this catalytically inactive form of the kinase could function in a trans-dominant negative mechanism. We found that transfections with the catalytically inactive K42A mutant protected cells from the IFN- $\gamma$ -induced cell death [67]. These studies closed a circle which started by the rescue of a death-protective anti-sense cDNA fragment, that 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, we proved that DAP-kinase is involved in cell death induced by TNF- $\alpha$  and by anchorage-independent growth [71]. Since the early signaling cascades generated by p55 TNF receptor or detachment from extracellular matrix do not share any known common elements with IFN- $\gamma$  signaling, DAP-kinase is likely to lie further downstream, and may function as a universal target at which different apoptotic signals eventually converge. Thus, the original design of the TKO approach, aimed at targeting elements that function downstream to early signaling, indeed proved to be beneficial.

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Fig. 4. The death-promoting function of DAP-kinase depends on the intrinsic catalytic activity. HeLa cells ( $5 \times 10^5$  cells/ml) were transfected with 20  $\mu\text{g}$  DNA of pcDNA3 vector, or with DAP-kinase constructs cloned in the same vector. After 48 h, the cell cultures were split 1:5 and subjected to selection with G-418. After 2–3 weeks the plates were stained with crystal violet.

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- $\gamma$  in HeLa cells [48]. Yet, we suspect that this may not be the major regulatory step. Preliminary data suggest that 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.

### 5.1.3. 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 and by application of cytoskeletal disrupting drugs [67]. 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 (Fig. 3). Most interestingly, cell killing by overexpression of DAP-kinase depended on its correct localization to the cytoskeleton. Expression of a truncated form of the kinase, catalytic active, yet mislocalized to the nucleus, failed to disrupt the actin microfilament and to kill the cells [67]. This was an important finding since loss of stress fibers and disruption of microfilament organization occur at early stages of epithelial and fibroblastic cell death. The specific intracellular localization of the kinase may therefore provide a mechanistic clue as to how external signals impose these cytoskeletal changes [67].

### 5.1.4. Implications of DAP-kinase in tumorigenesis

Positive mediators of cell death may be lost or inactivated in tumors and may therefore function as tumor suppressor genes, as clearly documented in the case of p53 (reviewed in Refs. [72,73]). Chromosomal localization studies mapped DAP-kinase to human chromosome 9 band q34.1 [74] — a region prone to translocations in human leukemias [75–77], and to LOH in bladder carcinomas [78,79]. 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 (Fig. 5), and in 30%

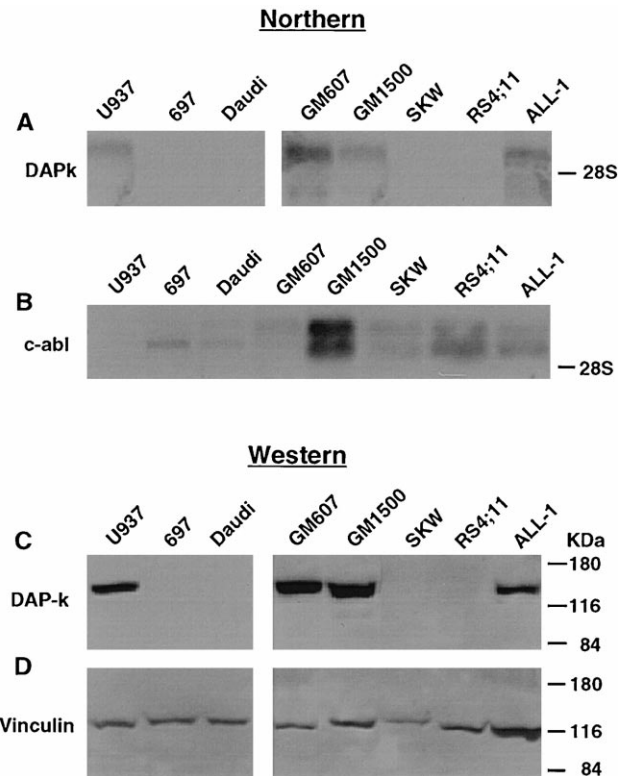


Fig. 5. Analysis of DAP-kinase expression in various hematopoietic cell lines. (a) and (b), Northern blot analysis of poly A<sup>+</sup> RNA from various cell lines using probes for DAP-kinase and c-Abl, respectively. (c) and (d), Western blot analysis of DAP-kinase protein and vinculin (as unrelated protein reference), respectively.

of cell lines derived from bladder carcinomas, breast carcinomas, and renal cell carcinomas [80]. 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. 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 and Jaenisch [81]). 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 recently carried out a second approach, that directly tested in animal models, whether the DAP-kinase gene has tumor suppressor functions. 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 [71]. 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 normal pattern of 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 vivo* 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 [71]. 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.

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 [71]. 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 [71]. These results provided the first indication implicating the DAP-kinase gene in augmenting the threshold sensitivity of the tumor cells to apoptotic signals. 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- $\alpha$  and anchorage-independent cell growth. The transfectants displayed higher sensitivity to the apoptotic effects of TNF- $\alpha$ : 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, we suggested that DAP-kinase-mediated suppression of metastasis results, at least in part, from increased sensitivity to various death-inducing stimuli. We propose that loss of DAP-kinase expression provides a novel mechanism that links suppression of apoptosis to metastasis.

## 5.2. *DAP-1 and DAP-3*

In this review, only a few selected aspects of DAP-1 and DAP-3 study will be highlighted. More details on these genes are available in Refs. [48,60].

DAP-1 was identified as a gene that encodes for a basic, proline rich protein (15 kDa), which carries a potential site for phosphorylation by cdk. Two

closely migrating forms of DAP-1 protein were detected in HeLa cells; the slowly migrating form corresponds to phosphorylated DAP-1 since it could be eliminated by *in vitro* phosphatase treatment [48]. 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 increased the killing of cells by IFN- $\gamma$  (T. Raveh and A. Kimchi; unpublished results).

DAP-3 gene encodes a 46 kDa protein carrying a potential P-loop motif, thus 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 tissues [60]. Ectopic expression of DAP-3 from a constitutive promoter induced massive death of HeLa cells, leading to a significant reduction in the number of drug resistant stable clones [60]. The gene is widely expressed in various cells and tissues [60]. Chromosomal localization studies mapped DAP-3 to human chromosome 1 band q21 [82]. By cloning the DAP-3 homologue from *C. elegans* we found that it shares 35% identity and 64% homology with the full length human protein. Not surprisingly, the murine DAP-3 protein is highly homologous to the human protein, and altogether these studies suggest that this gene has been highly conserved during evolution.

### 5.3. DAP-5

Analysis of the deduced amino acid sequence of the full length 97 kDa DAP-5 protein, revealed that it is highly homologous to the translation initiation factor 4G (eIF4G; p220) (overall 27% identity and 48% similarity). Yet, it differed from p220 in some interesting features, as detailed below and in Ref. [62]. This finding focused our subsequent interest on a subject that was so far poorly addressed in the field of programmed cell death: possible alterations of the protein translation machinery under apoptotic stress.

Initiation factor 4G, frequently called p220, is part of the cap binding complex (for a review see, Ref. [83]). It functions as a scaffold for assembling several initiation factors that direct ribosomes to RNA at the cap site. The N-terminal part of p220 binds eIF4E, the well known cap-binding protein, whereas the middle region of the protein binds eIF3 and eIF4A initiation factors (see Fig. 6). Interestingly, alignment of the newly isolated DAP-5 to p220 indicated that this novel protein lacks the N-terminal region of p220 responsible for the association with the cap-binding protein eIF4E (Fig. 6). This information was very important in light of the finding that during lytic infection by RNA viruses, a specific proteolytic cleavage of the N-terminal region of p220 abrogates its binding to the capped cellular mRNAs [84,85]. As a consequence, the cap-dependent translation of cellular RNAs stops, and the cleaved p220 directs transla-

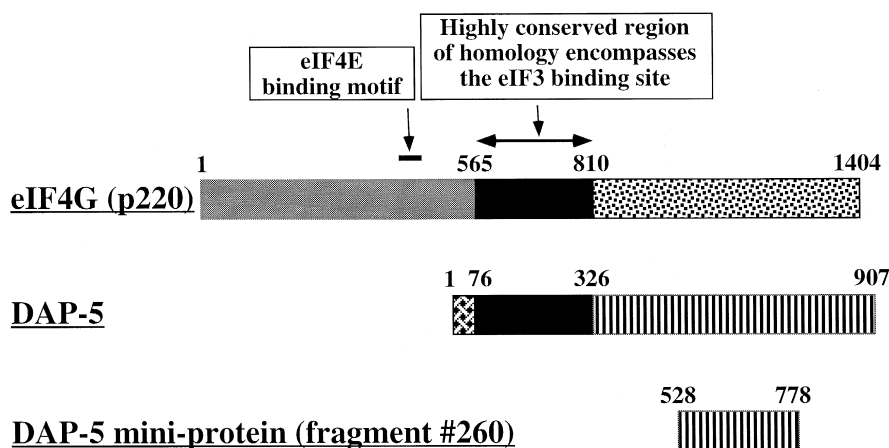


Fig. 6. Schematic representation of DAP-5 protein aligned to p220 translation factor. The most conserved region of homology is marked by a black box; the other parts show less, yet significant homology, as described in the text. The position of the rescued death-protective mini-protein is shown. The numbers indicate the amino acid positions.

tion from the uncapped viral mRNAs by binding to internal ribosome entry sites, named IRES [86]. In this respect, DAP-5 is a naturally occurring protein which structurally resembles the proteolytically cleaved p220, suggesting that it will fail to support cap-dependent translation, and raising a challenging possibility that it may also share some other functional properties with the truncated p220 form. Indeed, the N-terminal part of DAP-5 shares high homology with the central region of mammalian p220, i.e., 39% identity and 63% similarity. This further suggests that some of the functions of p220, residing in the middle part of p220 protein, such as binding to eIF-3 or eIF-4A initiation factors, have been conserved. The C-terminal region, on the other hand, is significantly less homologous to the corresponding region of p220, suggesting that DAP-5 may also possess unique functional properties, not present in p220 [62].

Surprisingly, the rescued DAP-5 cDNA fragment, which conveyed resistance to IFN- $\gamma$ -induced cell death, was expressed from the vector in the sense orientation. In retrospect, we realize that internal fragments of cDNAs were cloned into the pTKO-1 expression vector in both orientations during the construction of our library. Sense fragments may be rescued during the selection in case they encode a truncated protein which confers growth advantage during IFN- $\gamma$ -induced cell death. Indeed, it was found that a mini-protein of 28 kDa in size was generated by this partial cDNA fragment, the translation of which started from an internal ATG present at the 5' end of this partial cDNA [62]. Intriguingly, this mini-protein corresponds to the less conserved C-terminal part of the full length DAP-5 protein (Fig. 6), suggesting that it may specifically affect DAP-5 functions. The product of the cDNA fragment could operate in a trans-dominant negative manner by inhibiting the association of the endogenous DAP-5 protein with its potential effectors, or by preventing interactions with activators that may be required for promoting the apoptotic effects of endogenous DAP-5.

Once expressed in different cells, the partial cDNA fragment displayed dual effects on cells, which provided an explanation for type II resistance. Low expression levels weakly protected the cells from IFN- $\gamma$ -induced programmed cell death, while en-

forced high expression levels were toxic to cells [62]. If the mini protein prevents interaction of full length endogenous DAP-5 with its downstream effectors, the dual effects imply that DAP-5 protein is essential for both cell viability and cell death. Thus, complete inactivation of the protein by excess of the mini-protein may be not compatible with cell viability (unlike DAP-1 to 3), and therefore stable transfectants express only low levels giving rise to a weak death-protected phenotype (type II resistance; Table 1). The alternative possibility is that the mini protein which covers the C-terminal part of DAP-5, prevents a putative activation step of the endogenous protein. In this scenario, DAP-5 full length protein, which is normally expressed at high levels (N. Levy and A. Kimchi, unpublished results), may be kept in an inactive form in growing cells, and be specifically activated at the post-transcription/translation level in response to an apoptotic stimulus. For example, the C-terminal region of the full length DAP-5 may negatively regulate the protein function (in cis or in trans by binding to an inhibitor), and the inhibition could be relieved either in a regulated manner during cell death, or artificially by large excess of the mini-protein in the transfectants. Thus, the high expression levels of the mini-protein may cause cell death by releasing the inhibitory effects of the C-terminal region and activating the apoptotic functions of the full length DAP-5, while low growth-permissive levels may partially prevent the activation of DAP-5 by the apoptotic stimulus, resulting in weak death-protective effects. Obviously, experiments are currently being performed to find out which one of these challenging working hypotheses are correct, each of which should highlight different intriguing aspects in DAP-5 mode of action.

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 by co-immunoprecipitation and in vitro binding experiments that DAP-5/p97 protein binds to eIF3 and eIF4A and that like the cleaved p220, it fails to bind the cap-binding protein eIF4E [87]. Assays based on transient transfections showed basic functional differences between p220 and DAP-5/p97. While the overexpression of p220 promoted both cap-dependent and cap-independent translation, the DAP-5/p97 overexpression re-

pressed both cap-dependent and cap-independent translation from viral IRESs. Based on these experiments, as well as on studies involving the inducible expression of this protein in stable HeLa clones, Imataka et al. proposed that DAP-5/p97 may function as a repressor of translation that titrates out the eIF3 and eIF4A from cells. An alternative working model proposes that DAP-5/p97, in its putative death-promoting active form, may drive cap-independent translation of certain critical cellular mRNAs, under stress conditions that limit cap-dependent translation. Whether cellular IRESs exist that can bind DAP-5/p97 remains to be determined, as discussed in a recent mini review [88].

Two additional lines of research recently ended up, quite surprisingly, in the independent cloning of DAP-5 gene. These studies introduced unexpected fascinating aspects in the study of this gene. Yamanaka et al., [89] developed an elegant strategy to clone novel targets for the RNA editing enzyme, named Apobec-1. Apobec-1 is the catalytic subunit of the editosome complex which substitutes cytidine to

uridine by cytidine deaminase activity. This mechanism can alter the encoded sequence of the protein and even create premature stop codons, as shown in the case of Apolipoprotein B [89] and Neurofibromatosis type I [90]. The target gene which they have cloned turned out to be DAP-5, and multiple sites along the DAP-5 RNA that undergo specific editing were subsequently mapped using an artificial system of hyperediting in transgenic mice [89]. DAP-5 mRNA contains multiple conserved motif sequences that are sufficient to induce editing in *in vitro* systems. This work opens up a novel research direction dealing with the possibility that Apobec-1-mediated DAP-5 RNA editing, may regulate DAP-5 function during apoptosis.

A second group has recently cloned the murine DAP-5 while searching, by positional cloning, for a gene that lies at the proximity of inserted retroviral sequences causing myeloid leukemia in mice [91]. It still remains to be determined whether the retroviral integration may negatively or positively influence the expression of DAP-5.

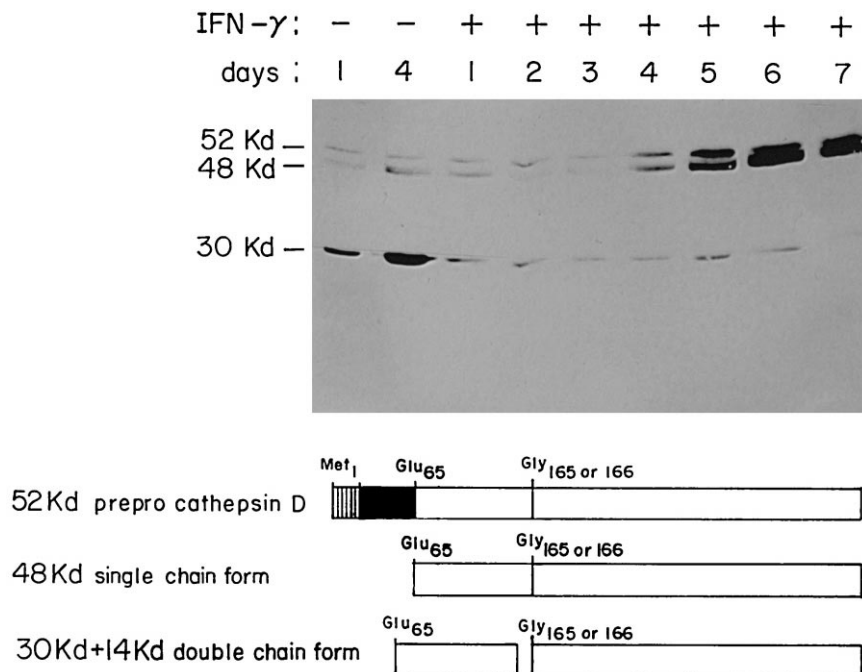


Fig. 7. Regulation of expression and processing of cathepsin D protease by IFN- $\gamma$ . Immunoblot analysis of cathepsin D forms before and after treatment with IFN- $\gamma$  (1000 U/ml). Cell extracts were prepared at the indicated time points from HeLa cells. Samples of 300  $\mu$ g were fractionated on SDS-PAGE (12%), blotted to nitrocellulose, and reacted with anti-cathepsin D antibodies. The sizes of cathepsin D forms are shown. A scheme that depicts the different steps of cathepsin D processing is shown below the immunoblot.

## 6. The cathepsin D protease connection to programmed cell death

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 [61].

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- $\gamma$  and Fas/APO-1-induced death. The phenotype fell into type I resistance, as shown in Fig. 2. 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- $\gamma$  and to the anti-Fas/APO-1 agonistic antibodies. Protection by pepstatin A was also detected during the TNF- $\alpha$ -induced programmed cell death of U937 cells [61]. We thus established the role of this protease in three different apoptotic systems.

The next challenge was to understand how a house-keeping 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 (see Fig. 7). 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, Wu et al. [92] 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 [92]. 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, as detailed below.

## 7. Concluding remarks concerning the genes which were rescued by the TKO technology

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 dominant-negative mutations (e.g., K42A DAP-kinase mutant), or used small peptide inhibitors (e.g., pepstatin A), to illustrate by a few independent approaches the rate limiting functions that these genes display in apoptosis. In addition, overexpression of several full length wild-type cDNAs (DAP-kinase, DAP-3, and cathepsin D) induced cell death in the

absence of any external stimulus. In many cases, 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 if the normal regulation of the gene during apoptosis does not necessarily involve elevation of expression. Examples include p53 [93], the various members of the caspase gene family [94–97] and the death domain-containing proteins that function proximal to Fas and the p55 TNF receptors [52–58]. The ectopic expression of DAP genes therefore provides an independent support for these genes being positive mediators of cell death, and will be used in the future as convenient functional assays for studying possible interactions with other apoptotic genes (see below).

The ubiquitous expression of these genes in many cell types and tissues, and the high degree of conservation among different organisms (so far shown for DAP-kinase, DAP-3, and DAP-5), imply that we managed to isolate evolutionary conserved and important components of cell death pathways that are common to various cells. Moreover, the spectrum of apoptotic stimuli that converge into DAP-genes and cathepsin D is very wide.

Regulation of DAP genes by the initial apoptotic signals is another issue which deserves close attention. If a gene that was initially rescued through its rate limiting function during cell death becomes modified by the apoptotic signal, directly or indirectly (at the transcriptional/post-transcriptional/post translational levels), then it can undoubtedly be considered as an intrinsic part of an apoptotic pathway. The best example obtained so far is the pattern of cathepsin D regulation during cell death which consists of a few independent levels, as detailed in Section 6. Obviously, we are currently investigating the issue of DAP genes regulation (DAP-1 to DAP-5) during apoptosis.

Finally, the link to cancer and to other pathologies is an important issue in evaluating the physiological role of DAP genes. The best indications that we have so far relate to the DAP-kinase gene which possesses strong anti-metastatic activities in experimental systems. We found that loss of DAP-kinase conveys to the metastasizing cells strong positive selection to resist various apoptotic stresses [71]. Obviously, human cancer specimens should be tested for genomic rearrangements in the near future, a prerequisite for

classifying the gene as a bona fide tumor suppressor gene.

Intriguingly, the genes which were rescued code for proteins that have a diverse spectrum of biochemical activities including a cytoskeleton-associated calcium/calmodulin-dependent kinase, a nucleotide binding protein, an aspartyl lysosomal protease, and a novel translation initiation regulator. The major challenge now is to analyze 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. Caspases could function in parallel to the biochemical pathways which involve DAP genes, or alternatively may be integrated into these pathways either as upstream modulators or as downstream effectors.

## **8. The growing field of functional approaches of gene cloning applied in mammalian systems: where did it lead us and future prospects**

As molecular biology is entering into the era of human functional genomics, it is clear that novel concepts leading to feasible molecular tools which screen genes according to their specific function (rather than their structure) will be required. A lot of emphasis was recently put on the development of technologies which are based on identification of differentially expressed genes. This includes PCR-based methods (RDA), or screens made by DNA chips or SAGE [98,99]. Yet, these approaches have two major serious limitations. First, they fail to discriminate between the functionally relevant genes

that are rate limiting in the process, and the other many differentially expressed genes, which are secondary in their importance. Second, they ignore an important set of genes which are being regulated during the biological process at the post-translational/transcription level (e.g., by protein phosphorylation or other covalent modifications, proteolytic cleavages, changes in protein intracellular localization, in interactions with other proteins, etc.).

In principle, functional approaches of gene cloning, which are based on random gene inactivation, followed by the selection of the desired phenotype, hit directly relevant rate limiting genes. If the phenotypic screen is based on positive growth selection, and is being performed in an appropriate background in which the spontaneous phenotypic changes are rare, then this approach becomes very powerful. Yet, it should be mentioned that a major limitation of the approach is that some of the selected rate limiting activities may eventually turn out to interact indirectly with the regulated pathways, rather than being intrinsic components which transmit the signal. Regulation studies may therefore help to resolve the issue. As previously discussed, if the rescued gene displays a typical pattern of regulation in response to the trigger, the chances are high that it is an intrinsic component. In general, the approach is suitable for the cloning of genes mediating different growth restrictive signals, including, in addition to apoptosis, scenarios of cell cycle arrest, cellular senescence, or suppression of tumorigenicity.

Several other versions of functional gene cloning, besides TKO, which are based on positive growth selection, were developed over the past few years leading to the isolation of intriguing novel genes. The general principles of these methods resemble the basis of the TKO strategy, yet they differ in some parameters, according to the different growth restrictive systems which were employed. These strategies were developed by Gudkov and Roninson [100], Gabig et al. [101], Vito et al. [102], and Li and Cohen [103]. Some of these approaches were applied in systems of programmed cell death triggered by cytotoxic drugs, growth factor deprivation, or T cell receptor activation. Others were applied in systems that assay for anchorage-independent growth in soft agar, or tumor formation in nude mice. They led to recent discovery of a few genes that have a role in

apoptosis (e.g., ALG-2, ALG-3, Requiem, kinesin heavy chain) as well as to isolation of two novel genes with tumor suppressive activity (TSG101, p33<sup>ING1</sup>).

One of the strategies of functional gene cloning, recently developed by Li et al. [104], was designed to search for retroviral integration sites in which the generation of anti-sense RNA disrupts expression of the corresponding genes from both alleles. The positive growth selection used in their system was the ability of certain mouse fibroblasts to grow in soft agar. This function was considered to be associated with cellular transformation and ability to form metastatic tumors in nude mice, as was later further confirmed experimentally. Using this approach they rescued a novel gene, with a potential tumor suppressor activity, named *tsG101* [103]. The human homologue, TSG101, was mapped to chromosome 11, bands 15.1–15.2, a region proposed to contain tumor suppressor genes. An initial analysis of human breast cancers by Southern analysis of PCR amplified DNA, suggested the presence of large intragenic deletions [104]. Yet, another recent more comprehensive screen of human breast cancer specimens, performed directly on genomic DNA, failed to detect any genomic rearrangements in the TSG101 gene [105]. Thus, while there is still a debate in the literature as of whether TSG101 gene is a bona fide tumor suppressor gene, the success of this approach in rescuing a gene that suppresses tumorigenicity in experimental model systems, extends the potential use of functional gene cloning to other selection systems.

Another functional strategy, named GSE (for genetic suppressive elements), was initially developed by I.B. Roninson, and applied successfully for the isolation of both apoptotic and growth inhibitory genes together with A.V. Gudkov. The strategy is based on the selection of short DNA fragments which are isolated from expression libraries of randomly fragmented cDNAs which are being expressed both in the sense and anti-sense directions [100]. As previously discussed, high expression of anti-sense RNAs could block protein production, while expression of truncated sense fragments could block function or activation of the corresponding protein in a dominant negative manner. The system was applied first in cells that are induced to undergo apoptotic cell death in response to the anticancer drug, etoposide. A few

GSEs were isolated, one of which encodes anti-sense RNA for the heavy chain of the motor protein, kinesin. Interestingly, expression of the kinesin anti-sense RNA induced resistance to several DNA-damaging drugs, and conferred growth advantage to senescent mouse embryo fibroblasts [106].

A recent work by Garkavtsev et al. [107], applied the GSE technology in *in vivo* selections for tumorigenicity. In this work, the GSE library was prepared from cDNA enriched with sequences that are being preferentially expressed in normal cells compared to their transformed counterparts. They have cloned a novel gene, named p33<sup>ING1</sup>, that displays tumor suppressive functions. The overexpression of this gene inhibited cell growth while the selected GSE that directed short anti-sense RNA promoted cell transformation in cultures [107]. In a recent work coming from Gudkov's laboratory, it has been shown that p33<sup>ING1</sup> cooperates with p53 in cell growth inhibition, and that this novel gene is a component of the p53 signaling pathway by modulating p53-dependent transcriptional activation. A physical interaction between p33<sup>ING1</sup> and p53 has been documented in this work by immunoprecipitation [108].

A third group employed a similar selection system that was named 'death trap'. They introduced cDNA expression libraries into T cell hybridoma cells induced to undergo apoptosis by T cell receptor cross linking. They isolated two genes involved in apoptosis. One, ALG-2, codes for a calcium-binding protein, and the other ALG-3, was identified as a homologue of the familial Alzheimer's disease gene STM2 [102,109,110].

It should be stressed that one of the invaluable advantages of functional approaches of gene cloning is that they are completely unbiased. As a consequence, novel targets and unpredicted mechanisms may be discovered. For example, the rescue of DAP-kinase highlighted a cellular target in apoptosis that so far was poorly addressed, and might be as important as the nuclear alterations, *i.e.*, the disruption of the actin microfilament system. These coordinated microfilament-dependent cell shape changes, which are mediated by DAP-kinase, now reflect critical causative steps that constitute the program of apoptosis. Consistent with this idea is the previous finding that GAS-2, a component of the microfilament system, is a substrate for caspases during cell death, and

that its specific proteolytic cleavage generates a GAS-2 form that induces alterations of the actin cytoskeleton and cell morphology characteristic of apoptosis [111].

DAP-5 studies, on the other hand, open a novel direction concerning possible alterations in the translational machinery during cell death. The concept was raised long ago, yet was not addressed on the molecular level ever since. It is clear that while some apoptotic systems are very rapid and occur in the presence of cycloheximide, many others develop much more slowly, depending on specific changes in the profile of protein translation, and are inhibited by cycloheximide. One of the studied examples is the metamorphic death of the tobacco hornworm labial glands, where a drop in protein synthesis, coupled to selective up regulation of a few other specific proteins, take place [112]. Similar changes in the pattern of translation were found in nerve growth factor-deprived sympathetic neurons [113]. In other types of cellular stresses such as heat shock, the majority of cellular proteins is suppressed by the elevated temperature, due to reduction of eIF4E function, while the synthesis of heat shock proteins remains refractory to this change, suggesting again a regulated pattern of protein translation. The discovery of DAP-5 may introduce the first molecular handle in understanding this important death-related control system of translation.

Altogether, the abovementioned examples represent the beginning of an era where functional gene cloning approaches, applied in mammalian cells, will provide a huge momentum to the molecular understanding of basic biological processes. Along with the completion of the human genome project, special focus should be put on elucidating the function of genes, and on understanding how do they constitute biochemical networks which impose the outcome of cellular phenotype.

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