

DAP Kinase—A Proapoptotic Gene That Functions as a Tumor Suppressor

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APOPTOSIS AND CANCER

Having a major impact on tumor initiation, progression, and metastasis, apoptosis has become a subject that draws tremendous attention and research efforts in the cancer field. At various stages during tumor development, cells are subjected to stressful conditions that trigger programmed cell death, and thus mutations leading to inhibition of apoptosis confer a selective advantage to cells. In premalignant cells, activation of oncogenes and the consequent hyperproliferation provoke a cellular response that leads to elimination of those cells by apoptosis. Subsequently, transformed cells in the tumor microenvironment are under constant selective pressure, such as lack of oxygen (hypoxia), depletion of growth/survival factors, attacks by the immune system, and often death by anoikis due to loss of cell–matrix interactions. At later stages, when metastasizing tumor cells enter into circulation they encounter many additional death-inducing signals such as superoxides, nitric oxides, killing cytokines, and mechanical shearing forces. Thus, all along the multistage process of tumorigenesis induction of apoptosis functions as a tumor-suppressor mechanism and cells have to escape from various inducers of apoptosis in order to survive (reviewed in Kaufmann and Gores, 2000; Lowe and Lin, 2000; Wyllie *et al.*, 1999). This means that tumor cells should benefit from mutations that either inactivate various intracellular proteins which positively mediate programmed cell death or activate antiapoptotic genes.

The first example which established the concept that genes in the apoptotic machinery are mutated in cancer was documented with the cloning of Bcl-2. The initial findings that this gene resides at the site of (8;14) chromosomal translocation characteristic of follicular B cell lymphoma (Tsujiimoto *et al.*, 1984) were followed by the elegant studies performed in transgenic mice models which altogether established a role for Bcl-2 activation in promoting cell survival and *in vivo* lymphomagenesis (McDonnell *et al.*, 1989;

Strasser *et al.*, 1990). The second well-established example is the p53 gene whose proapoptotic functions have been thoroughly studied ever since they were first documented (Yonish-Rouach *et al.*, 1991). Inactivating mutations of p53 are frequently found in a wide range of human tumors. The inactivation of p53, by deletions or mutations, reduces the sensitivity of cells to apoptosis triggered by oncogene activation, hypoxia, telomere erosion, changes in cell adhesion, and DNA-damaging agents, thus providing a powerful positive selection at the different stages of tumor development (reviewed in Gottlieb and Oren, 1998). These two well-studied examples provided the milestones for establishing the link between apoptosis and cancer.

In light of the complexity of the molecular network of apoptosis and the diversity of stress signals operating in the multistep process of tumorigenicity, it became of interest to look for additional apoptotic genes which may be involved in cancer development. Therefore, when death-associated protein (DAP) kinase was first isolated in our laboratory as a positive mediator of apoptosis, one of the most exciting questions was to find out whether it may function as a tumor-suppressor gene. This article is devoted to recent studies which investigated from different angles the possible link between DAP kinase and cancer.

DEATH-ASSOCIATED PROTEIN KINASE—A POSITIVE MEDIATOR OF PROGRAMMED CELL DEATH

DAP kinase was identified in our laboratory as a result of long-lasting efforts to isolate novel positive mediators of programmed cell death by genetic selection in mammalian cell cultures. To this end we developed a function-based gene cloning methodology coupled to powerful positive growth selections (Deiss and Kimchi, 1991). This methodology, named technical knockout screen, was based on random inactivation of genes by expression of an antisense cDNA library followed by selection of clones that survived in the continuous presence of an apoptotic stimulus. Interferon- γ (IFN- γ), which kills HeLa cells very efficiently, was chosen as the initial apoptotic stimulus. In this system, specific inhibition of DAP-kinase protein expression by antisense mRNA

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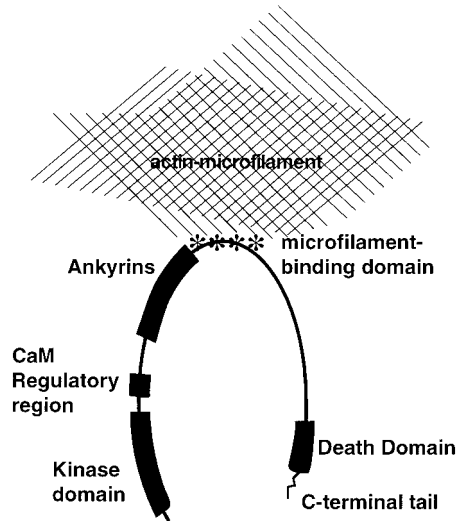


FIG. 1. Schematic representation of DAP-kinase protein. The various motifs and domains are shown. The crossed lines represent the actin-microfilament network.

protected HeLa cells to some extent from programmed cell death induced by IFN- γ (Deiss *et al.*, 1995). It is important to note that the strategy was designed to specifically hit the genes which function downstream of the IFN- γ early signaling complex, in order to increase the probability of cloning genes which rather belong to the basic machinery of cell death. This was confirmed later on by showing that DAP-kinase dependence is not exclusive to IFN- γ and is common to other stress signals, including those which are encountered by tumor cells during cancer development (see below).

The DAP-kinase gene codes for a Ca²⁺/calmodulin-regulated serine/threonine kinase that is found localized to the cytoskeleton, specifically in association with the actin microfilaments (Cohen *et al.*, 1997). This 160-kDa protein displays an interesting multidomain structure that is very unique among members of the extended family of calmodulin-dependent protein kinases (Hanks and Quinn, 1991). The protein-kinase domain resides at the N-terminus and is followed by a segment comprising the calmodulin (CaM) binding and regulatory domains. In addition, the protein carries eight ankyrin repeats, a cytoskeleton binding region, and a conserved death domain (Feinstein *et al.*, 1995). The death domain is followed by a serine-rich stretch of amino acids residing at the C-terminus of the protein (Fig. 1). The eight ankyrin repeats, as well as the death domain, may mediate interactions with putative effector proteins or influence the specificity and/or stability of kinase-substrate interactions.

The multidomain structure of DAP kinase and its participation in a wide range of apoptotic systems (Cohen *et al.*, 1999) imply that this protein may interact with various intracellular components to exert its ac-

tion. *In vitro*, DAP kinase phosphorylates itself and the myosin light chain (MLC). One of the main challenges in this respect is to identify the entire range of DAP kinase's physiological substrates and to understand how their phosphorylation impinges upon the biochemical pathways through which DAP kinase executes its proapoptotic actions. One direction is now emerging from a work which showed that MLC phosphorylation is essential for the process of membrane blebbing in apoptosis (Mills *et al.*, 1998).

The death-promoting function of DAP kinase is regulated by at least two distinct autoinhibitory mechanisms. The enzyme's active site is inhibited by the adjacent CaM regulatory domain, and this inhibition is relieved upon binding of Ca²⁺/CaM. Accordingly, deletion of the calmodulin regulatory domain generated a constitutively active kinase (DAPk- Δ CaM) with an enhanced ability to induce apoptosis in cultured cells (Cohen *et al.*, 1997). A second mode of autoinhibition was revealed in a genetic screen, based on functional selection of short DAP-kinase-derived fragments which could protect cells from apoptosis by acting in a dominant-negative manner. One short fragment comprised the serine-rich C-terminal tail, spanning the last 17 amino acids of the protein. It was characterized as an autoinhibitory module since deletion of this tail from the full-length kinase increased the death-inducing activity, without affecting the catalytic activity, potentially by preventing its intramolecular folding over other functional domains (Raveh *et al.*, 2000). DAP kinase is therefore kept silent in viable cells due to these two independent self-restraining mechanisms. Activation of DAP kinase during apoptosis involves the release from these negative autoregulatory mechanisms, and in some cases it also involves an increase in the protein levels (see below).

Ectopic expression of DAP kinase in cultured cells of various types was shown to trigger cell death. A catalytically inactive mutant of DAP kinase, generated by substituting a lysine essential for ATP binding by alanine (K42A), failed to induce cell death, thus establishing the importance of the kinase domain for the proapoptotic effects. Moreover, this mutant inhibited the activity of the wild-type protein by functioning in a dominant-negative manner (Cohen *et al.*, 1997). In addition to the catalytic activity, the death domain was found to be essential for apoptosis induction, and transfections with this module by itself protected cells from various death-inducing signals, by specifically neutralizing the function of the endogenous DAP kinase (Cohen *et al.*, 1999). Using these two forms of dominant-negative mutants, DAP kinase was shown to modulate death induced by interferon- γ , Fas, TNF- α , detachment from extracellular matrix, and oncogene activation (Cohen *et al.*, 1999; Deiss *et al.*, 1995; Inbal *et al.*, 1997; Raveh *et al.*, 2001), indicating its wide

involvement in apoptosis. Taken together with the abundant pattern of expression in different tissues (Yamamoto *et al.*, 1999), these findings point at the possible involvement of DAP kinase in various physiological scenarios in which elimination of cells is carried out.

Obviously, one of the challenges was to find out whether this proapoptotic gene is a potential tumor suppressor subjected to loss-of-function mutations in cancer. To this end, a few independent directions were undertaken in the past few years, based on functional assays and tumor screens. The functional assays were performed both in cell cultures and in mouse model systems, which altogether assessed the potential activity of this protein in suppressing different stages of tumorigenicity. In parallel, the status of the gene in human tumor specimens was analyzed and correlations were made with respect to the aggressiveness of the disease in several cases. These three independent lines of research are summarized below.

DAP KINASE IN ONCOGENE-INDUCED p53 ACTIVATION—CHANNELING p53 TOWARD APOPTOSIS

One of the first apoptotic checkpoints in the multi-step process of tumorigenicity is turned on early during transformation of primary cells in response to unbalanced hyperproliferative signals. The latter can be imposed by the aberrant activation of oncogenes such as c-Myc, E1a, and E2F-1 or by loss of Rb function (Bates *et al.*, 1998; de Stanchina *et al.*, 1998; Pomerantz *et al.*, 1998; Zindy *et al.*, 1998). Induction of apoptosis at this stage is critical for the elimination of oncogene-bearing cells, and in many systems it is being carried out in a p53-dependent manner (Lowe, 1999). Oncogene-induced activation of p53 is mediated by p19ARF—a regulator of p53 stability that is necessary for responses to mitogenic signals and not for responses to DNA damage (Sherr, 1998). Thus, it appears that a critical step in the onset of cancer is oncogene activation and that the ability of p53 to respond to this alteration by induction of apoptosis is a rate-limiting determinant of its tumor-suppressor function. Consistently, tumor formation in p19ARF-deficient mice greatly resembles that of mice lacking p53 (Donehower *et al.*, 1992; Kamijo *et al.*, 1997), and in tumors of mouse and human origin mutations in p19ARF and p53 are usually mutually exclusive (reviewed in Sharpless and DePinho, 1999). Thus, although the functions of p19ARF and p53 do not overlap completely, they do operate along the same antioncogenic pathway.

Once the various aspects in the antitumorigenic functions of DAP kinase started to emerge in our laboratory it became of interest to investigate whether DAP kinase specifically operates in this early apoptotic checkpoint. A basic finding that initiated the interest

in this direction was the strong suppression that activated DAP kinase exerted on transformation of primary fibroblasts by Myc and Ras or E1A and Ras (Raveh *et al.*, 2001). Most importantly, this inhibition of foci formation by the introduction of activated DAP kinase was completely abrogated in p53^{-/-} fibroblasts, indicating that functional p53 was absolutely required. A detailed molecular dissection of the process revealed that DAP kinase increased the p53 protein levels as well as its p53-responsive genes in a p19ARF-dependent manner. Moreover, the p53-dependent reduction in foci number resulted from DAP-kinase-induced apoptosis which involved caspase activation and DNA degradation (Raveh *et al.*, 2001). At least two domains within DAP kinase were required for getting the full apoptotic/focus-suppressive effects, i.e., the intact catalytic activity and the presence of the death domain.

The finding that the ectopically expressed DAP kinase killed primary mouse embryo fibroblasts in a p19ARF/p53-dependent manner immediately raised the following intriguing questions: Does it reflect the involvement of the endogenous DAP kinase in the well-studied safeguard mechanism that is turned on in response to oncogenes? Is the endogenous DAP kinase one of the missing components that couples oncogenes to p19ARF/p53 activation and is responsible for channeling these fibroblasts toward apoptosis?

The subsequent experiments indeed confirmed that the endogenous DAP kinase functions in this specific apoptotic checkpoint to modulate p53 responses. First, it was found that endogenous DAP-kinase protein is up-regulated in response to c-Myc or E2F-1 activation. Second, and most importantly, the inactivation of DAP kinase either functionally by a dominant negative mutant or genetically by targeted mutation attenuated the apoptotic responses to c-Myc or E2F-1. The targeted mutation was achieved once the DAP-kinase-deficient mice were established in our laboratory. The responses of the DAP-kinase-null primary fibroblasts to oncogenes were compared to those of the wild-type counterparts. In DAP kinase^{-/-} fibroblasts, the oncogene-induced rise in p53 and the extent of apoptosis were both attenuated (Raveh *et al.*, 2001). These results imply that DAP kinase is an intrinsic component along the p19ARF/p53 pathway that is activated by oncogenes to induce apoptosis (Fig. 2).

It is noteworthy that the mere activation p19ARF, by overexpression in normal fibroblasts, leads to cell cycle arrest (Quelle *et al.*, 1995), whereas activation of p19ARF/p53 by oncogenes sensitizes the cells to apoptosis. This difference suggests that oncogenes induce additional changes in cells and that those putative changes functionally interact with p53 to induce apoptosis. The mechanisms responsible for channeling p53 toward induction of apoptosis rather than cell cycle arrest are still unknown. Our studies provide the first

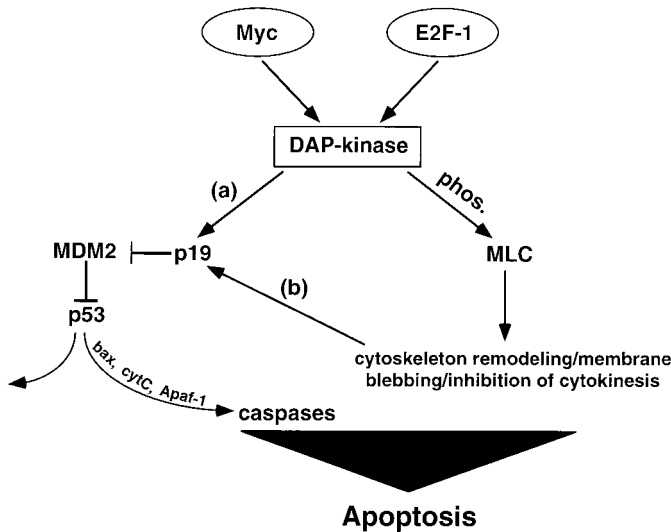


FIG. 2. DAP kinase mediates an oncogene-induced apoptotic checkpoint in fibroblasts. Dereglulation of c-Myc or E2F-1 leads to increase in DAP-kinase protein levels. Activated DAP kinase has two independent functional arms. One is wired to p19ARF/p53, resulting in induction of p53-responsive genes. The second affects cytoskeleton possibly via MLC phosphorylation. Activation of each arm alone is not sufficient to induce cell death in fibroblasts and only the convergence of the two pathways leads to caspase activation and apoptosis. Arrows (a) and (b) point to two possibilities through which DAP kinase impinges upon the p19ARF/p53 pathway.

example of a protein kinase that is up-regulated by c-Myc or E2F-1 and takes part in forming the appropriate cellular “context” that channels the activated p53 toward apoptosis (Raveh *et al.*, 2001). Experiments performed in fibroblasts have shown that both p19ARF and DAP-kinase expression was required for induction of apoptosis. These results imply that aside from activation of p53, other DAP-kinase-mediated events should cooperate with the former to induce apoptosis (see Fig. 2). In fibroblasts, this additional, p53-independent pathway is not sufficient to cause cell death by itself, yet its collaboration with the p19^{ARF}/p53-dependent arm of DAP-kinase activity appears to be essential for triggering apoptosis. (It should be noted that in various carcinoma cells and other cell lines, this p53-independent arm is sufficient by itself to induce killing.) The conclusion from these studies is that when induced by oncogenes, DAP kinase contributes both to activation of p53 and to creating a cellular environment that dictates the preferential induction of p53-dependent apoptosis.

The precise biochemical nature of the p53-independent pathway activated by DAP kinase is not known. Notably, when MEFs were infected with activated DAP kinase and maintained for up to 7 days in the presence of a selective drug, a fraction of the cells that did not undergo apoptosis displayed 4N and higher DNA content. Microscopic examination revealed an increase in

the frequency of multinucleated cells. In p53- and p19ARF-deficient MEFs, which failed to undergo apoptosis in response to the activated DAP kinase, there was a more prominent appearance of multinucleated cells. About 30–50% of cells appeared with 4N DNA content as early as 48 h after transfection (Tal Raveh and Adi Kimchi, unpublished observations). This phenomenon is likely to reflect activation by DAP kinase of a cellular pathway which regulates cytokinesis and becomes evident in cells that survive DAP-kinase overexpression or, more remarkably, once the p19^{ARF}/p53 pathway is impaired. The biochemical mechanism underlying this phenomenon is currently unknown. However, our recent finding that DAP kinase phosphorylates myosin light chain on serine 19, a site which is relevant to myosin II activation (Shani Bialik and Adi Kimchi, unpublished results) lends support to the hypothesis that the kinase inhibits cytokinesis via MLC phosphorylation. Thus, the process of MLC phosphorylation by DAP kinase, which may lead to cytoskeletal remodeling/inhibition of cytokinesis and/or to membrane blebbing (all of which are caspase-independent events) may contribute indirectly to p53 channeling toward induction of apoptosis. The wiring of DAP kinase to p19ARF may emerge from its capability to phosphorylate cytoskeletal components or may involve instead another substrate, either the components of the p19ARF/MDM2/p53 complex themselves or other targets that interact with them (see the model in Fig. 2).

DAP KINASE AND METASTASIS—AFFECTING LATE STEPS IN TUMORIGENESIS

A survey of cancer cell lines derived from various human tumors has shown that the mRNA and protein expression of DAP kinase were frequently lost (Kissil *et al.*, 1997). It was found in this respect that DAP-kinase mRNA and protein expression levels were below detection limits in 80% of B cell lymphoma and leukemia cell lines and in 30–40% of cell lines derived from bladder carcinomas, breast carcinomas, and renal cell carcinomas (Table 1). This stood in contrast to the finding that DAP-kinase mRNA is ubiquitously expressed in immortalized cell lines established from nontumorigenic cell sources and in normal tissues and primary cell cultures. This initial screen provided the first hint that DAP-kinase expression may be lost in the course of malignant transformation.

An interesting paradigm emerged once mouse lung carcinoma cell lines from different sources, which are being used in experimental metastasis assays, were studied. It was found that while all the nonmetastatic clones deriving from Lewis lung carcinoma still expressed normal levels of DAP kinase, the highly metastatic clones were all DAP-kinase negative. Subse-

TABLE 1

The Status of DAP Kinase in Human Tumors and Human Cancer Cell Lines

Type of tumor	(%)	Aberration	Reference
B cell lymphoma	(84%)	DNA methylation	Katzenellenbogen <i>et al.</i> , 1999
Non-small cell lung cancer	(23%)	DNA methylation	Esteller <i>et al.</i> , 1999
Head and neck cancer ^a	(18%)	DNA methylation	Sanchez <i>et al.</i> , 2000
Non-small cell lung cancer ^b	(44%)	DNA methylation	Tang <i>et al.</i> , 2000
Thyroid lymphoma	(84%)	DNA methylation	Nakatsuka <i>et al.</i> , 2000
Colon carcinoma	(26%)	DNA methylation	Kissil and Kimchi (unpublished)
Colon and breast cancers	(15%)	LOH	Kissil and Kimchi (unpublished)
Tumor cell lines with no DAPk expression			
B cell leukemia	(80%)		Kissil <i>et al.</i> , 1997
Breast carcinoma	(30%)		Kissil <i>et al.</i> , 1997
Bladder carcinoma	(29%)		Kissil <i>et al.</i> , 1997
Renal carcinoma	(40%)		Kissil <i>et al.</i> , 1997

^a Hypermethylation predicts lymph node metastasis.

^b Hypermethylation predicts disease-specific 5-year survival.

quently, DAP-kinase expression was restored by transfection in the highly metastatic clones, yielding clones expressing the transgene at physiological levels, with no changes in the overall pattern of their growth *in vitro*. The metastatic activity of these genetically manipulated cells was examined by intravenous injection of the cells into syngeneic mice. Strikingly, restoration of physiological levels of DAP kinase into the highly metastatic Lewis carcinoma cells suppressed their ability to form lung metastases in mice (Inbal *et al.*, 1997). Both the number and the size of lung lesions were strongly reduced or even completely eliminated. Conversely, rare lung lesions which were spontaneously selected in mice following injection of the original poorly metastatic cells had lost endogenous DAP-kinase expression at high frequency. Altogether, these experiments suggested that loss of DAP-kinase expression provides a positive selective advantage during the formation of lung metastases. The transgene also delayed local tumor growth, yet with lower efficiency than the suppression of the metastatic activity (Inbal *et al.*, 1997).

The mechanisms underlying the suppressive effects of DAP kinase on metastasis and local tumor growth were then 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. These results indicated that expression of the DAP-kinase transgene re-sensitized the tumor cells to apoptotic stimuli, a property which they had originally managed to circumvent by losing DAP kinase. To directly address this point, the transfected cells were exposed *in vitro* to different apoptotic stimuli of the sort they may encounter during

metastasis. Stimuli that trigger apoptosis during the process of metastasis formation include lack of survival signals delivered by cell adhesion, cytokines secreted by cells of the immune system, and residence in a foreign tissue exposed to a growth factor repertoire different from in the original environment (Fidler, 1991). It was found that reintroduction of DAP kinase conveyed sensitivity to TNF- α or to detachment from extracellular matrix, whereas DAP kinase deficient counterparts were capable of growing under anchorage-independent conditions (Inbal *et al.*, 1997). Based on these experiments, it has been suggested that DAP-kinase-mediated suppression of metastasis results, at least in part, from increased sensitivity to various death-inducing stimuli. These experiments proposed that loss of DAP-kinase expression confers a selective advantage on cancer cells and may play a causative role in tumor progression toward the more aggressive metastasizing cells (Inbal *et al.*, 1997).

In conclusion, the *in vivo* and *in vitro* function-based assays described above attributed tumor-suppressive properties to DAP kinase at at least two different apoptotic checkpoints which operate in the course of tumor development. These assays propose that loss of DAP kinase may confer a selective advantage during the early oncogene-activated apoptotic checkpoint, by attenuating the p53 responses, and during the late stages of metastasis by reducing the sensitivity of the metastasizing cells to detachment from extracellular matrix. The next step from here was to make *in situ* screens to test the status of the DAP-kinase gene in fresh human tumor specimens. This important project was undertaken simultaneously by a number of groups in the past year, demonstrating altogether loss of DAP-kinase expression in many types of cancer as summarized below.

LOSS OF DAP-KINASE EXPRESSION IN HUMAN TUMOR SPECIMENS

An observation which we made a few years ago in the cancer cell lines which we screened facilitated some of the subsequent *in situ* measures of DAP kinase in cancer patients. We found in this respect that in some (but not all) of the DAP kinase⁻ cell lines, DAP-kinase expression could be restored by the DNA-demethylating drug 5-aza-2'-deoxycytidine (Kissil *et al.*, 1997). This indicated that aberrant DNA methylation was one of the mechanisms responsible for turning off this gene. Later, a CpG island at the 5'UTR of DAP kinase was found to be a potential target for hypermethylation. The biological relevance of this methylation was demonstrated in the Burkitt's lymphoma cell line Raji, in which DAP kinase is fully methylated and not expressed. In these cells, the demethylation by treatment with 5-aza-2'-deoxycytidine restored their apoptotic sensitivity to IFN- γ (Katzenellenbogen *et al.*, 1999).

Promoter methylation is considered the main epigenetic modification in mammals, and abnormal methylation of the CpG island in the promoter regions of genes leads to transcriptional silencing. Other tumor-suppressor genes that are known to be affected by methylation include p16 INK4a, p15 (INK4b), p14 (ARF), Von Hippel-Lindau, and retinoblastoma (Laird, 1997). The methylation status of DAP kinase's 5'UTR was analyzed in DNA extracted from primary normal and tumor samples, using methylation-specific polymerase chain reaction (MSP) developed by Herman and his co-workers.

The MSP method is based on chemical modification of cytosine to uracil by sodium bisulfide treatment. In these reactions all cytosines are converted to uracils, but those that are methylated are resistant to this modification and remain as cytosine. Primers specific for either the methylated or the modified unmethylated DNA are then used for DNA amplifications. Methylation in CpG sites is scored by gel fractionations of the amplified DNA and detection of positive signals with the corresponding primers. The primer sequences for DAP kinase were derived from the 5' region of the gene, which contains a typical GpC island. All the normal tissues had unmethylated copies exclusively.

This analysis revealed high incidence of hypermethylation in B cell malignancies (Katzenellenbogen *et al.*, 1999), thyroid lymphoma (Nakatsuka *et al.*, 2000), non-small cell lung carcinoma (Esteller *et al.*, 1999; Tang *et al.*, 2000), and head and neck cancer (Sanchez-Céspedes *et al.*, 2000). Using the same technique, we found in our laboratory that 26% of tested breast and colon carcinoma cancers displayed methylation of DAP-kinase CpG island (J. Kissil and A. Kimchi, unpublished data) (see Table 1). The highest frequency of DAP-kinase methylation was detected in B cell lym-

phoma. All Burkitt's lymphomas tested and 84% of B cell non-Hodgkin's lymphomas tested were hypermethylated in the DAP-kinase CpG island (Katzenellenbogen *et al.*, 1999). Two other studies had also linked DAP-kinase methylation status with disease prognosis. In head and neck cancer 18% of the tested patients displayed promoter methylation of DAP kinase. Within this group, hypermethylation of DAP kinase correlated with the presence of lymph node metastases and advanced disease stage. In non-small cell lung cancer (NSCLC), a retrospective study found hypermethylation of the DAP-kinase promoter in 44% of the tumors and a strong association between hypermethylation of the DAP-kinase promoter and poor disease-specific survival prospects. Strikingly, of the molecular markers assessed so far in NSCLC (including k-ras and p53 mutations), DAP-kinase hypermethylation was the only independent factor that predicted disease-specific survival rate (Tang *et al.*, 2000). These observations are in accordance with the role of DAP kinase in preventing tumor dissemination, as shown in the mouse model (Inbal *et al.*, 1997).

These multiple studies indicate altogether that the DAP-kinase gene is abnormally methylated in a significant portion of human tumors, providing the first proof that inactivation of this gene is critical for the development of these common malignancies. Yet, as is the case for the other tumor-suppressor genes, and as predicted from the 5-aza-2'-deoxycytidine experiments discussed above, DNA methylation is not an exclusive way to inactivate DAP kinase. Loss or inactivation may also result from mutations, deletions, or other DNA rearrangements. Two LOH cases were already identified in our initial screen of breast and colon carcinoma cancers once the DAP-kinase gene was mapped in our laboratory to chromosome 9, region q21.3-q22.3 (J. Kissil and A. Kimchi, unpublished data). Obviously the other DAP-kinase allele in these two samples should be screened for possible intragenic mutations. A summary of the status of DAP kinase in human tumors and cancer cell lines is shown in Table 1.

DISCUSSION AND PROSPECTS

The different lines of research which were performed so far indicate that loss of DAP kinase confers a selective advantage at different stages of cancer development, mainly due to its proapoptotic activity. Is DAP kinase a bona fide tumor-suppressor gene? The issue still awaits additional research work. First, the search for intragenic mutations and other genomic rearrangements should be extensively worked out in human cancers. The statistical values that will be collected over the next few years will determine, together with the promoter methylation screens, whether DAP kinase has a definitive prognostic value for evaluating

the aggressiveness of the disease in certain types of cancer. They will also evaluate the therapeutic potential of this gene, especially during the late metastatic stages.

The statistics concerning DAP-kinase aberrations should be worked out together with the information relating to the status of p53 and p19ARF in the same human tumors. We predict that a major prognostic difference will exist between the cases in which mutations/methylations of DAP kinase and of p53 are mutually exclusive and the cases harboring double mutations. While the former may reflect the disruption of a pathway where these genes are wired to each other (e.g., the oncogene-induced apoptotic checkpoint), the latter may indicate a disruption of the p53-independent arm of DAP kinase during metastasis. In this respect, it is noteworthy that the Lewis lung carcinoma clones that were used in the metastasis studies described above carry mutant p53 (V. Rotter, personal communication), indicating that the antimetastatic effect of DAP kinase in these cells is p53-independent. This further implies that having a mutation in p53 does not preclude the need to inactivate DAP kinase, because DAP kinase may participate in additional late checkpoints that are activated during the progression of cancer cells, regardless of p53 status.

The second line of research that should be developed relates to cancer development in the DAP-kinase-null mice. DAP-kinase knockout mice have now been generated and are viable and fertile. These mice will serve to investigate the causative role for DAP kinase loss in tumor initiation and progression. Studying the phenotype of DAP-kinase-deficient mice will complement and extend our previous work regarding the antioncogenic activity of DAP kinase and will open a new research avenue of analyzing the function of this gene *in vivo*. An appealing possibility stems from our cell culture-based experiments, which suggested the involvement of DAP kinase in the apoptotic response to deregulated c-Myc (Raveh *et al.*, 2001), and from the finding that DAP-kinase expression is lost in human B cell lymphoma at high frequency (Katzenellenbogen *et al.*, 1999). DAP kinase may be involved in B cell malignancies, which invariably carry aberrations of the c-Myc oncogene. To test the role of DAP kinase in lymphomagenesis *in vivo*, we shall initiate crosses of DAP-kinase knockout mice to E μ -Myc transgenic mice (Landgon *et al.*, 1986) and evaluate the rate and latency of tumor formation.

REFERENCES

- Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998). p14ARF links the tumour suppressors RB and p53. *Nature* **395**, 124–125.
- Cohen, O., Feinstein, E., and Kimchi, A. (1997). DAP-kinase is a Ca²⁺/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J.* **16**, 998–1008.
- Cohen, O., Inbal, B., Kissil, J. L., Raveh, T., Berissi, H., Spivak-Kroizaman, T., Feinstein, E., and Kimchi, A. (1999). DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. *J. Cell Biol.* **146**, 141–148.
- de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S. Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev.* **12**, 2434–2442.
- Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O., and Kimchi, A. (1995). Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* **9**, 15–30.
- Deiss, L. P., and Kimchi, A. (1991). A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. *Science* **252**, 117–120.
- Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215–221.
- Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. (1999). Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res.* **59**, 67–70.
- Feinstein, E., Wallach, D., Boldin, M., Varfolomeev, E., and Kimchi, A. (1995). The death domain: A module shared by proteins with diverse cellular functions. *Trends Biochem. Sci.* **20**, 342–344.
- Fidler, I. J. (1991). Cancer metastasis. *Br. Med. Bull.* **47**, 157–177.
- Gottlieb, T. M., and Oren, M. (1998). p53 and apoptosis. *Semin. Cancer Biol.* **8**, 359–368.
- Hanks, S. K., and Quinn, A. M. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* **200**, 38–62.
- Inbal, B., Cohen, O., Polak-Charcon, S., Kopolovic, J., Vadai, E., Eisenbach, L., and Kimchi, A. (1997). DAP kinase links the control of apoptosis to metastasis. *Nature* **390**, 180–184.
- Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91**, 649–659.
- Katzenellenbogen, R. A., Baylin, S. B., and Herman, J. G. (1999). Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* **93**, 4347–4353.
- Kaufmann, S. H., and Gores, G. J. (2000). Apoptosis in cancer: Cause and cure. *BioEssays* **22**, 1007–1017.
- Kissil, J. L., Feinstein, E., Cohen, O., Jones, P. A., Tsai, Y. C., Knowles, M. A., Eydmann, M. E., and Kimchi, A. (1997). DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: Possible implications for role as tumor suppressor gene. *Oncogene* **15**, 403–407.
- Laird, P. W. (1997). Oncogenic mechanisms mediated by DNA methylation. *Mol. Med. Today* **May**, 223–229.
- Landgon, W. Y., Harris, A. W., Cory, S., and Adams, J. (1986). The c-myc oncogene perturbs B lymphocyte development in E μ -myc transgenic mice. *Cell* **47**, 11–18.
- Lowe, S. W. (1999). Activation of p53 by oncogenes. *Endocr. Relat. Cancer* **6**, 45–48.
- Lowe, S. W., and Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis* **21**, 485–495.

- McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., and Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57**, 79–88.
- Mills, J. C., Stone, N. L., Erhardt, J., and Pittman, R. N. (1998). Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J. Cell Biol.* **140**, 627–636.
- Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlov, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92**, 713–723.
- Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**, 993–1000.
- Raveh, T., Berissi, H., Eisenstein, M., Spivak, T., and Kimchi, A. (2000). A functional genetic screen identifies regions at the C-terminal tail and death-domain of death-associated protein kinase that are critical for its proapoptotic activity. *Proc. Natl. Acad. Sci. USA* **97**, 1572–1577.
- Raveh, T., Droguett, G., Horwitz, M. S., DePinho, R. A., and Kimchi, A. (2001). DAP-kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat. Cell Biol.* **3**, 1–7.
- Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., Jen, J., Herman, J. G., and Sidransky, D. (2000). Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res.* **60**, 892–895.
- Sharpless, N. E., and DePinho, R. A. (1999). The INK4A/ARF locus and its two gene products. *Curr. Opin. Genet. Dev.* **9**, 22–30.
- Sherr, C. J. (1998). Tumor surveillance via the ARF-p53 pathway. *Genes Dev.* **12**, 2984–2991.
- Strasser, A., Harris, A. W., Bath, M. L., and Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by co-operation between myc and bcl-2. *Nature* **348**, 331–333.
- Tang, X., Khuri, F. R., Lee, J. J., Kemp, B. L., Liu, D., Hong, W. K., and Mao, L. (2000). Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J. Natl. Cancer Inst.* **92**, 1511–1516.
- Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., and Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226**, 1097–1099.
- Wyllie, A. H., Bellamy, C. O., Bubbs, V. J., Clarke, A. R., Corbet, S., Curtis, L., Harrison, D. J., Hooper, M. L., Toft, N., Webb, S., and Bird, C. C. (1999). Apoptosis and carcinogenesis. *Br. J. Cancer* **80**(Suppl. 1), 34–37.
- Yamamoto, M., Takahashi, H., Nakamura, T., Hioki, T., Nagayama, S., Ooashi, N., Sun, X., Ishii, T., Kudo, Y., Nakajima-Iijima, S., Kimchi, A., and Uchino, S. (1999). Developmental changes in distribution of death-associated protein kinase mRNAs. *J. Neurosci. Res.* **58**, 674–683.
- Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* **352**, 345–347.
- Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.* **12**, 2424–2433.

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