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Review: Spotlight on Cancer

DAPk Protein Family and Cancer

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ABSTRACT

The Death-Associated Protein kinase (DAPk) family contains three closely related serine/threonine kinases, named DAPk, ZIPk and DRP-1, which display a high degree of homology in their catalytic domains. The recent discovery of protein-protein interactions and kinase/substrate relationships among these family members suggests that the three kinases may form multi-protein complexes capable of transmitting apoptotic or autophagic cell death signals in response to various cellular stresses including the misregulated expression of oncogenes in premalignant cells. Several lines of evidence indicate that the most studied member of the family, DAPk, has tumor and metastasis suppressor properties. Here we present an overview of the data connecting the DAPk family of proteins to cell death and malignant transformation and discuss the possible involvement of the autophagic cell death-inducing capacity of DAPk in its tumor suppressor activity.

INTRODUCTION

A functional screen, based on random knockdown of gene expression, was developed in our laboratory in the past decade with the aim of isolating new genes involved in cell death. This technique, called the "technical knock-out" (TKO), is based on the transfection of an antisense cDNA library into HeLa cells and on the selection of clones that survive in the presence of continuous cytotoxic signals induced by interferon-γ.[1] Isolation and characterization of various cell death-protective antisense cDNA fragments which were rescued by this screen led to the discovery of a large group of new genes which function as positive mediators of cell death pathways. Hence the new genes obtained by this technique were named "Death-Associated Protein" or DAP genes.[2] One of the genes isolated by the TKO approach encoded a calcium calmodulin regulated serine/threonine kinase, and was named DAP kinase (DAPk1 or simply, DAPk).[3]

Further studies showed that, in fact, DAPk was the prototype of a novel protein family involved in apoptotic and autophagic cell death, tumor suppression and metastasis suppression. In this review, we will first briefly introduce the DAPk family of proteins and their role in apoptotic and autophagic cell death, and then discuss in depth, the accumulating clinical, experimental and molecular data connecting the DAPk protein family to tumor and metastasis suppression.

DAPk FAMILY OF PROTEINS: INTRODUCTION TO THE CLOSEST THREE MEMBERS

In addition to DAPk, the DAPk protein family includes at least two other closely related kinases, DAPk2/DRP-1 (DRP-1 for DAPk-related protein. Not to be confused with dynamin-related protein also commonly abbreviated as DRP-1) and DAPk3/ZIPk/Dlk (ZIPk) sharing around 80% identity in their kinase domains.[4,5] Interestingly, the extra-catalytic domain structures differ substantially from each other, suggesting that there should be some regulatory and functional divergence among these family members (Fig. 1; for more structural details see refs. 6 and 7). Nevertheless, all three kinases were shown to induce cell death upon their overexpression in cells. DRAK1 and DRAK2 are two other protein kinases, which display homology to DAPk; yet they are more distantly related (sharing only 50% identity in their catalytic domains with the other three members) and have been less well characterized. Therefore, in this review, we will restrict the use of the term "DAPk proteins" to DAPk, ZIPk and DRP-1.

Although DAPk was initially discovered due to its role in cell death induced by interferon-γ, DAPk family members were later shown to mediate cell death triggered by

several other death-inducers. Accumulating data indicate the involvement of these kinases in cell death induced by various stimuli, including TNF-α, Fas ligand, ceramide, oncogenes, TGF-β, arsenic trioxide and detachment from extracellular matrix (Fig. 2A).[8-15]

A complex hierarchy of interactions, including kinase-substrate relationships seems to exist among the DAPk proteins. Although the picture is still far from complete, a dynamic map of cross interactions has emerged from data obtained so far (Fig. 2B). DRP-1-induced death signals require DAPk function, since DRP-1-induced cell death was attenuated by the coexpression of a dominant negative mutant of DAPk.[4] Furthermore, DAPk was shown to phosphorylate ZIPk at several sites, which were mapped in the extra-catalytic domain. Phosphorylation of ZIPk by DAPk led to cytoplasmic retention and increased the death-inducing capacity of ZIPk.[16,17] Strikingly, these three kinases are capable of physically interacting with each other in cells. Quite unexpectedly, it was found that DAPk and ZIPk interact with each other through their catalytic domains and that a unique basic loop located in the kinase domain is responsible for this interaction.[16] It is currently assumed that these kinases form a "death-associated multi-protein complex", possibly together with other so far unknown regulatory proteins, translating, modulating and amplifying various stress signals to death-related outcomes (see ref. 16; Shani G, Kimchi A, unpublished data).

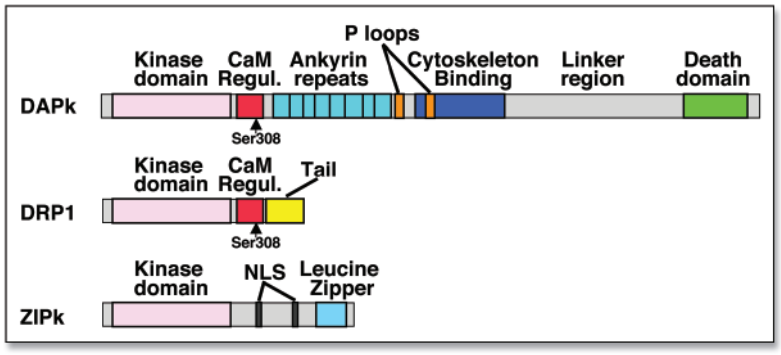

Figure 1. DAPk family of death-inducing kinases. DAPk is the largest protein consisting of several domains with various functions. Besides the kinase domain, DAPk protein also contains a death domain and ankyrin repeats which may mediate its interaction with other proteins. A cytoskeleton-binding region is responsible for DAPk intracellular localization to actin microfilaments. While DRP-1 is mainly cytosolic, ZIPk contains nuclear localization signals (NLS) and shuttles between the cytosol and nucleus. DAPk and DRP-1 share a calcium/calmodulin-binding regulatory domain (CaM Regul.) that is not present in ZIPk. Therefore, these two kinases sense, and are activated by a rise in cytosolic calcium concentrations resulting from cellular stresses, through binding of calcium-activated calmodulin. In addition to the calmodulin-dependent regulation, autophosphorylation of Ser308 in the calmodulin-binding domain of DAPk and DRP-1 maintain these kinases in an inactive state in healthy cells. ZIPk and DRP-1 may also be regulated by oligomerization through their "leucine zipper" or C-terminal tail (Tail) sequences, respectively.

THE FUNCTIONAL LINK OF DAPk FAMILY MEMBERS TO APOPTOSIS AND AUTOPHAGY

It has been shown over the years that the type of cell death induced by the ectopically expressed DAPk family of kinases depends on cellular and experimental context (Fig. 2A). In some models such as primary fibroblasts, overexpression of the DAPk family members induced caspase-dependent cell death with apoptotic characteristics like DNA fragmentation and caspase activation.[11,12,18] However, in other cells, e.g., HeLa, MCF-7 or HEK 293 cells, overexpression of DAPk proteins led to a caspase-independent cell death.[19] Closer analysis of DAPk and DRP-1-induced morphological changes by electron microscopy revealed that HEK 293 or MCF-7 cells transfected with these kinases contain in their cytoplasm many autophagic vesicles and autolysosomes.[19] Furthermore, overexpression of DAPk, DRP-1 or ZIPk led to autophagic membrane association of the otherwise soluble autophagy marker GFP-LC3 (see ref. 16; Gozuacik D, Kimchi A, unpublished data). Therefore, all three members of the DAPk family are involved, under certain circumstances, in a non-apoptotic cell death with accumulation of autophagic vesicles.

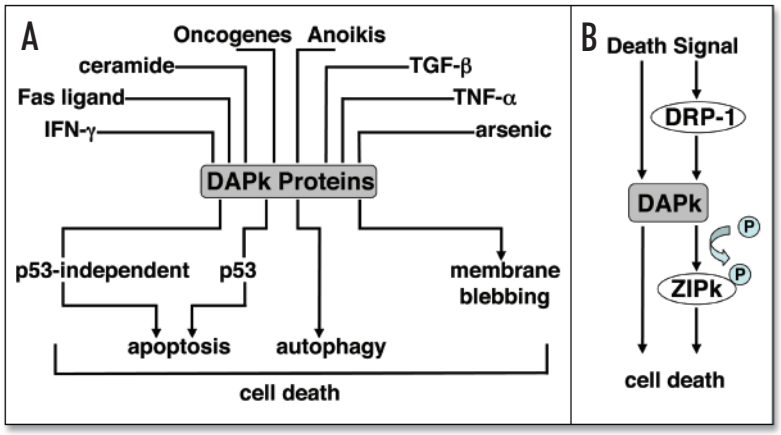

Figure 2. DAPk signalling cascade. (A) Upstream signals leading to DAPk family activation and the cellular outcomes. Various cell death stimuli may activate DAPk proteins. This may lead to the p53-dependent or -independent activation of apoptosis or to the activation of autophagy. Phosphorylation of the myosin light chain by DAPk proteins leads to membrane blebbing, another hallmark of programmed cell death. (B) Interrelationships between DAPk proteins. Overexpression of a dominant negative DAPk attenuates DRP-1-induced cell death, indicating that DRP-1 may function upstream to DAPk. Phosphorylation of ZIPk by DAPk leads to its cytoplasmic retention and increase in its death-inducing capacity. Nevertheless, alternative upstream and downstream pathways may still exist.

This type of nonapoptotic cell death, earlier classified by Clarke[20] as autophagic type II cell death (type I being the apoptotic cell death), was long ignored by the majority of the scientific community, and its very existence as an alternative and universal cell death phenomenon was underestimated until recently. This is mainly due to the fact that while autophagy seems to have both survival and death related roles in cells,[21,22] only one side of this duality was intensively documented, i.e., the survival-related role of autophagy in yeasts and higher organisms, especially under limiting nutrient conditions (for a comprehensive list, see ref. 22). Furthermore, until recently, causal relationships with cell death were missing, and any possible link to cell death was purely correlative. More direct evidence for the existence of autophagic cell death was only recently substantiated by an increasing number of studies in mammalian cells which showed that knock-out or knock-down of autophagy regulating proteins, like Atg5, Atg7 or Beclin 1, attenuated a non-apoptotic

form of cell death proceeding with an increased autophagic activity.[23-26] Moreover, Beclin 1/Atg6, which initiates autophagy in yeast and mammalian cells, triggers a non apoptotic cell death upon its release from its inhibitory association with Bcl-2.[26] This recent series of experiments, taken together with our initial finding that kinases from the DAPk family, which are potent death-promoting proteins, trigger the autophagy process, strongly support the existence of autophagic cell death. How the accumulation of autophagic vesicles contributes to cell death still remains an open issue (see ref. 21 for a detailed discussion). Yet, in the specific settings where autophagy is lethal to cells, it is legitimate to assume that failure to induce autophagic cell death when necessary might contribute to cancer development.

Although some overlap may exist, individual DAPk family proteins may have independent or complementary roles in autophagy pathways. While DAPk is necessary for interferon-γ-induced autophagy and cell death, it seems not to be a crucial element in starvation or rapamycin-induced autophagy (Gozuacik D, Inbal B, Kimchi A, unpublished data). In contrast, the overexpression of a dominant negative form of DRP-1 was able to attenuate amino acid/serum starvation or steroid withdrawal-induced autophagy in MCF-7 cells.[19] Additionally, electron microscopy analysis with immunogold staining revealed that overexpressed DRP-1 protein, but not DAPk accumulated in the lumen of autophagic vesicles.[19] Therefore, DRP-1 may play an additional role in the general autophagy machinery and its kinase activity may be involved in this function.

Overall, these observations establish the DAPk family of proteins as stress-activated proteins that connect cellular stresses like interferon-γ exposure, starvation or growth factor deprivation to autophagy pathways and to autophagic type II cell death. The involvement of members like DAPk or ZIPk also in caspase-dependent apoptotic cell death,[11-13,15,18] suggests that these death-associated kinases may act as "molecular switches" or "integration points" between apoptotic and autophagic cell death pathways.

DAPk FAMILY AND CANCER: A NOVEL TUMOR SUPPRESSOR FAMILY OF PROTEINS?

A common epigenetic change in human cancers: DAPk promoter methylation. A survey of the mRNA and protein expression of DAPk in various tumor cell lines led to the seminal discovery that a significant fraction of cell lines derived from human B cell neoplasms, bladder, breast and renal cell carcinomas have lost DAPk expression.[27] Strikingly, a detailed analysis showed for the first time that, in most of the cases, the loss of expression was not due to a deletion/rearrangement of the DAPk gene, but due to the epigenetic silencing of the gene by methylation. Indeed, the treatment of some cell lines that do not express DAPk protein with 5'-azadeoxycytidine (an agent causing the removal of methyl groups from CpG nucleotides) restored DAPk expression.[27] Gene silencing by methylation is a well-documented mechanism in tumorigenesis and loss of expression of tumor suppressor genes like p16, VHL and Rb by promoter methylation has been demonstrated in various human cancers.[28] Additional analysis of primary tumor samples from patients with colon cancer revealed that the DAPk gene was methylated in 26% of these tumors.[29]

Following those initial observations, more than 100 reports from different laboratories were published on DAPk gene methylation in various human tumors. Because the detection of methylation in a certain region of the DAPk gene by methylation-specific PCR correlated well with the loss of expression of DAPk mRNA and protein in several studies, the majority of the published work used this technique to analyze DAPk status in tumors. DAPk gene methylation has been observed in more than 20 types of human cancers originating from various tissues, including lymphomas and leukemias, lung, breast, colon, cervix, prostate cancers, and brain tumors. Therefore, loss of DAPk expression by cancer-specific gene methylation is a general phenomenon observed in the majority of human cancers and these observations implicate DAPk function in tumor suppression.

A microarray analysis of a small group of human squamous cell carcinoma samples revealed downregulation of ZIPk in these tumors. This indicates that, the tumor suppressor properties of ZIPk and DRP-1, like those of DAPk, deserve further attention in the future.[30]

Alternative mechanisms of DAPk inactivation in tumors. Hypermethylation of the DAPk promoter seems to be a major mechanism of DAPk inactivation in human tumors, but alternative mechanisms may exist, as to be expected for a bona fide tumor suppressor gene. Initial support for this emerged from the finding that treatment with 5'-azadeoxycytidine of some B cell and lung cancer cell lines did not restore DAPk expression.[14,27] The lack of DAPk expression in the absence of methylation has also been observed in some cases by others.[31,32] Indeed, we observed loss of heterozygosity as a mechanism of loss of DAPk expression in 15% of human colon and breast cancer specimens.[29] Additionally, homozygous deletion of DAPk was reported in pituitary tumors and soft tissue leiomyosarcomas (benign tumors of smooth muscle).[33,34] No study so far addressed the issue of possible hot spots of point mutation in DAPk family genes. Search for point mutations or small deletions, especially in functionally important domains like the kinase domain, should allow us to fully appreciate the importance of this protein family in human malignancies.

DAPk as a metastasis suppressor protein. The absence of DAPk expression in highly metastatic variants of mouse lung cancer cell lines, in contrast to its presence in the low metastatic variants of those same cell lines, led to the study of the role of DAPk expression in lung tumor growth and metastasis.[14] In an in vivo model of experimental metastasis, reintroduction of DAPk by stable transfection in the highly metastatic clones that lost DAPk expression, attenuated the metastatic capacities of these cells. The analysis of rare distant metastases arising from low metastatic clones showed that they regained metastatic capacity by losing DAPk expression. In in vitro tests, DAPk-expressing clones were more sensitive to cell death caused by TNF-α treatment or detachment from extracellular matrix, while their metastatic counterparts were relatively resistant. Additionally, tumor growth was delayed in DAPk-expressing clones and these tumors showed an increase in TUNEL-positive cells. These data introduced DAPk as a new metastasis suppressor protein that functions by increasing the sensitivity of tumor cells to programmed cell death during the multistep process of metastasis.

In line with these experimental results, clinical studies statistically correlated loss of DAPk expression with a more malignant tumor phenotype and increased metastatic capacity in human tumors. In lung cancers, DAPk promoter methylation was associated with aggressive disease and poor survival.[35-38] Lung tumors that were positive for DAPk hypermethylation showed advanced pathological stage, larger tumor size and lymph node involvement.[35] Similarly, DAPk hypermethylation in head and neck cancer correlated with lymph node involvement and advanced disease stage.[39,40] Analysis

of oligodendroglioma samples showed that the DAPk hypermethylation rate increased in parallel with the grade of malignancy of these tumors.[41] Additionally, loss of DAP-kinase expression in pituitary tumors, biliary tract tumors and urinary tract and gastric cancers and hepatocellular carcinoma was associated with advanced tumor stage and aggressive phenotype.[33,42-46] A survey of breast tumors revealed that DAPk hypermethylation was observed mostly in the invasive subtype of tumors and was associated with poor prognosis.[31,47] In brain metastases of various solid tumors, DAPk silencing by methylation was observed in the majority of cases.[48,49] Hypermethylation of the DAPk gene was significantly lower in primary cutaneous melanomas than metastatic ones.[50] All these data highly suggest a role for the DAPk protein as a metastasis suppressor protein in human tumors.

DAPk methylation as a cancer diagnosis tool. The frequent methylation of DAPk in human tumors in spite of its absence in the corresponding normal tissue led several groups to propose the use of DAPk methylation as a diagnostic and follow up test for several types of cancer. By analyzing DAPk methylation status, it was possible to detect urinary bladder cancer from voided urine; head and neck cancer from saliva, mouth rinsing fluid or nasopharyngeal swab or serum; colon and breast cancer from serum; lung cancer from sputum, bronchial brush samples or serum; and cervix cancer from cervical cytology specimens.[51-61] Consequently, DAPk downregulation by methylation is a cancer-specific event, which may be used to specifically detect malignant cells.

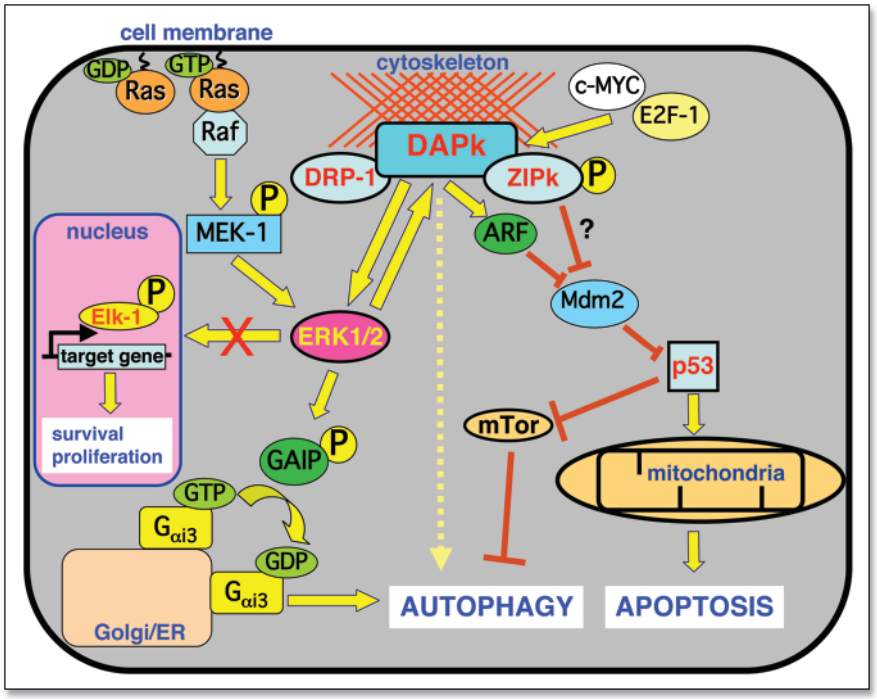

Figure 3. Schematic drawing summarizing molecular mechanisms mediating the action of the DAPk proteins in tumor suppression. See the text for details. ER, endoplasmic reticulum.

Molecular mechanisms. Data accumulated in recent years have begun to uncover the molecular mechanisms mediating the action of the DAPk family of proteins in tumor suppression (Fig. 3). The first insight into one of the molecular mechanisms came from the study of malignant transformation in primary rodent embryonic fibroblasts by foci formation tests.[11] In this classical test of oncogenic or tumor suppressor potential of genes, DAPk coexpression with the combination of oncogenes such as Myc and Ras, or E1A and Ras, led to suppression of transformation by these pairs of oncogenes. Strikingly, the transformation by the combination of T antigen with Ras was refractory to DAPk and regained sensitivity only if the p53-binding C-terminal portion of T antigen was deleted. Consistent with these data, p53 knockout fibroblasts were also resistant to the suppression of foci formation by DAPk, suggesting that the transformation suppressive activity of DAPk in this model system depends on p53 activity.

In non-transformed cells, in addition to their well-documented growth promoting activity, oncogenes like Ras, Myc or E2F-1 are wired to cell death and/or senescence pathways, forming "death or senescence checkpoints" which impose an additional level of regulation against uncontrolled cellular proliferation.[62] During malignant transformation, inactivation of these checkpoints is a common occurrence that serves to suppress apoptosis or senescence and to allow uncontrolled proliferation. One such death pathway induced by oncogenes involves the activation of the p53 pathway through induction of p19ARF.[63] ARF induction by oncogenes leads to stabilization of p53 by inactivating its negative regulator, Mdm2.

Strikingly, p53-dependent cell death induction by oncogenes like Myc and E2F-1 was attenuated after inactivation of DAPk by dominant negative mutants or by gene deletion.[11] DAPk overexpression stimulated p53's transcriptional activity in a p19ARF-dependent manner and DAPk-induced cell death in fibroblasts required the presence of both functional p53 and p19ARF genes. Therefore, one of the outcomes of loss of DAPk in cancer development may involve the attenuation of the p53-dependent proapoptotic signals triggered by oncogenes like c-Myc, thus contributing to the early steps of oncogenic transformation in vivo. Interestingly, in human breast tumor specimens, the epigenetic inactivation of the DAPk and p53 genes was mutually exclusive, supporting the idea that also in human tumors, DAPk inactivation may contribute to attenuation of p53-dependent cell death.[31]

Several major questions are currently under investigation concerning the cross talk between members of the DAPk family and the p53 pathway. One issue refers to the identification of the direct kinase substrate(s), the phosphorylation of which promotes p53 function in this system. Is p19ARF a relevant direct substrate? What is the functional implication of recent findings showing that Mdm2 binds to members of the DAPk family and that a peptide derivative of Mdm2 is phosphorylated by ZIPk on Ser 166?[64] Are there additional substrates in this pathway? Recent work by Feng et al. reveals a connection between p53 and the mTor pathway that regulates autophagy in response to amino acid and/or growth factor deprivation.[65] In this study, p53 activation stimulated autophagy through inhibition of mTor activity in an AMPK and TSC1/2 complex-dependent manner. DNA damaging agents such as etoposide, which activate the p53 pathway, were also shown to induce autophagic cell death in Bax/Bak double knockout cells.[25] In light of these data, it is possible that DAPk-induced autophagy may involve p53 activation in some cellular settings. Thus, the role of p53-induced

autophagy in the tumor suppressive functions of DAPk needs to be further studied.

Induction of p53-dependent apoptosis by DAPk indirectly, by the blockade of integrin signaling via an inside-out mechanism, has been proposed as an alternative mechanism of p53-induction by DAPk.[15] The presence of p53-binding sites in the DAPk promotor sequence, and induction of DAPk expression by p53, indicate that a positive feedback loop may be operational in which DAPk and p53 activate each other.[66] An additional connection between the DAPk family and the p53 pathway came from a phage-peptide display analysis, which led to the identification of ZIPk as an Mdm2 and p21-interacting protein.[64] ZIPk phosphorylated peptides from both interacting proteins, indicating the presence of another level of regulation of the p53 pathway by the DAPk family. Nevertheless, DAPk is also involved in p53-independent cell death in response to other types of stimuli; therefore, the contribution of disruptions in p53-independent pathways to tumorigenesis should also be considered.[12,19]

A recent study by Chen et al. revealed an interplay between DAPk and the ERK1/2 pathway.[67] The authors showed that DAPk physically interacted with ERKs and that ERK2 directly phosphorylated DAPk. The phosphorylation by ERK2 led to an increase in DAPk kinase activity and increased the cell death-promoting properties of the kinase. DAPk, in turn, promoted the cytoplasmic retention of ERK1/2 and thus interfered with the activation of ERK nuclear targets, such as Elk-1. Since the nuclear functions of ERK1/2 are mostly related to their capacity to promote cell survival and proliferation,[68,69] retention of ERK1/2 in the cytoplasm by DAPk may block those ERK-driven signals. Furthermore, activation of DAPk by cytoplasmic ERK1/2 may further stimulate death signals initiated by DAPk in an explosive manner, leading to the elimination of cells in which the Ras/Raf/ERK cascade was aberrantly activated. Loss of DAPk during tumorigenesis may contribute to oncogenic signals provided by the Ras/Raf/ERK pathway by abolishing this additional layer of ERK1/2 regulation and cell death checkpoint.

Interestingly, ERK2 was shown to directly phosphorylate and activate Golgi- localized, autophagy-inducing Gα-interacting protein (GAIP), a regulator of Gαi3-dependent macroautophagy in human colon cancer cells.[70] Indeed, the Ras/Raf/ERK pathway was shown to regulate autophagy induction through regulation of GAIP phosphorylation.[71] Thus, it is possible that cytosolic retention of ERK1/2 by DAPk contributes to the autophagy-inducing and/or tumor suppressor activity of the DAPk proteins. This hypothesis needs to be further tested.

CONCLUSIONS AND PERSPECTIVES

The extensive promoter methylation screens combined with the experimental data described in this paper have established the tumor suppressor functions of DAPk and highlighted its importance in the diagnosis and prognosis of cancer. From the molecular point of view, some of the well-documented pathways in the mode of action of DAPk, such as p53 activation or the cross-talk with ERK, highlight its function as a tumor suppressor gene. The detailed molecular mechanisms which link the different DAPk family members to autophagy still await further studies.

The widespread inactivation of DAPk proteins in human tumors and experimental evidence that introduction or activation of these kinases activates apoptosis and/or autophagy in cancer cell lines raises the possibility of using DAPk-based strategies (i.e., DAPk family gene therapy, development of activators of DAPk family function, use of demethylating agents to reestablish DAPk expression, etc.) as novel therapeutic modalities against cancer.

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