

DAP-kinase as a target for drug design in cancer and diseases associated with accelerated cell death

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Abstract

Misregulated cell death, which can result in either the excessive, inappropriate elimination of cells, or in the insufficient removal of damaged or malignant cells, has been associated with numerous diseases. Here we discuss an important molecular regulator of cell death, DAP-kinase (DAPk), which presents a promising target for therapeutic intervention. A structure-functional analysis of this calcium-regulated Ser/Thr kinase which promotes cell death will be presented, and emphasis will be placed on particular disease models in which its modulation might affect clinically-relevant cell death processes.

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1. Introduction

1.1. Programmed cell death and cancer

Apoptosis has become a subject that draws tremendous attention and research efforts in the cancer field, by virtue of its effects on tumor initiation, progression and metastasis. At various stages during the course of 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 pre-malignant cells, activation of oncogenes and the consequent hyper-proliferation 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 the circulation, they encounter many additional death-inducing signals, such as superoxides, nitric oxides, killing cytokines, and mechanical shearing forces. Thus, all along the multi-stage process of tumorigenesis, induction

of apoptosis functions as a tumor suppressor mechanism. In order to survive, tumor cells have to escape from various inducers of apoptosis, and would thus benefit from mutations that either activate anti-apoptotic genes or inactivate pro-apoptotic genes (reviewed in [1–3]).

The concept that genes in the apoptotic machinery are mutated in cancer was first established with the cloning and characterization of Bcl-2 [4,5]. The initial findings that this gene resides at the site of the (8;14) chromosomal translocation characteristic of follicular B cell lymphoma, were followed by elegant studies performed in transgenic mouse models which demonstrated a role for Bcl-2 activation in promoting cell survival and in-vivo lymphomagenesis [6,7]. The second tumor suppressor gene identified as an apoptosis regulator was p53, whose pro-apoptotic functions have been thoroughly studied ever since they were first documented [8]. 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 [9]). 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 underlying the different types of programmed cell death, and the diversity of stress signals operating in the multi-step process of tumorigenicity, it became of interest to look for

Abbreviations: CaM, calmodulin; CNS, central nervous system; DAPk, DAP-kinase; IFN- γ , interferon- γ ; MEFs, mouse embryonic fibroblasts; MLC, myosin II regulatory light chain; MLCK, myosin light chain kinase

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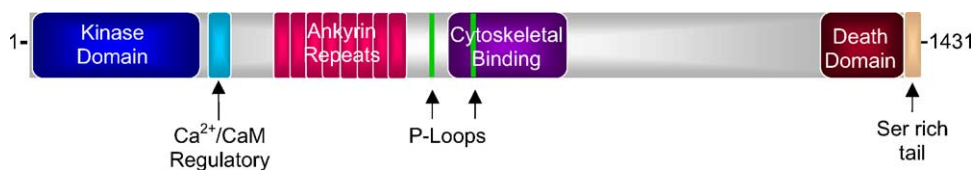


Fig. 1. Schematic diagram of DAPk protein structure. Shown are the known functional domains of DAPk. See text for details.

additional apoptotic genes which may be involved in cancer development. Therefore, when DAP-kinase (DAPk) was first isolated in our laboratory as a positive mediator of apoptosis, one of the most exciting questions was to discover whether it functions as a tumor suppressor gene. Here, recent studies investigating the possible link between DAPk and cancer, and experimental data showing how its loss or inactivation promotes tumorigenesis, will be presented. Importantly, an ever-increasing amount of clinical data gathered over the last 3 years indicates that DAPk is a possible drug target in cancer therapy. While the field is still in its premature stages, the exciting information on the mode of action of this relatively novel serine/threonine kinase opens up new directions in designing novel strategies in cancer therapy.

1.2. Neuronal cell death and DAPk

The other side of the coin concerning DAPk's structure/function analysis relates to its hyper-activation or gain of function abnormalities, which might lead to excessive cell death in some human pathologies associated with cell loss. Of special interest is the DAPk involvement in neuronal cell death. Mature neurons in adult organisms undergo apoptotic cell death in response to a variety of stress conditions, including lack of neurotrophic factors, anoxia, excitotoxicity, traumatic injury and neurodegenerative disorders. Obviously, deciphering the genes and proteins that constitute the molecular pathways which lead to neuronal cell death under pathological conditions provides new targets for drug design. Recent information about the activation of DAPk in hippocampal neurons by various stress signals and the determination of its participation in multiple death processes, raised the possibility of this kinase's involvement in pathologies of the nervous system. Furthermore, the advanced understanding of the protein's structure and mode of action that has been achieved to date will enable design of specific inhibitors of DAPk and allow for their potential clinical use, as will be discussed in this chapter.

2. What is known about DAPk's structure?

2.1. The discovery of a new death promoting kinase

DAPk was discovered in the mid 1990s in a genetic screen in which an antisense library was used to identify genes necessary for interferon (IFN)- γ -induced death in HeLa cells

[11]. Subsequent sequence and activity analysis indicated that DAPk encoded a Ca^{2+} /calmodulin (CaM) regulated Ser/Thr kinase, with a catalytic domain highly homologous to that of myosin light chain kinase (MLCK) [11–13]. This 160 kDa protein bears an interesting multi-domain structure, including ankyrin repeats and the death domain (Fig. 1). DAPk's necessity for cell death is not limited to IFN- γ signaling; numerous studies have since demonstrated that DAPk activity is required for the induction of cell death by multiple death signals, including those generated by death receptors, cytokines, matrix detachment, and hyperproliferation [13–16]. Furthermore, ectopic expression of the kinase is sufficient to induce pronounced death-associated cellular changes, which include membrane blebbing, cell rounding and the formation of autophagic vesicles [13–19].

A few years after the isolation of DAPk, additional family members which share high identity in their catalytic domains (80% identity at the amino acid level) were cloned and identified. The family includes DRP-1/DAPk2 and Dlk/ZIP-kinase [20–23]. The highly conserved nature of the catalytic domain accounts for several shared properties, including some common substrates and similar functional effects. Yet, the three family members differ in their extra-catalytic protein domains, which confer unique activities and subcellular localizations to each kinase. As yet, only DAPk has been linked to particular death-related pathologies, and, as such, will be the focus of this chapter. Nevertheless, when designing specific drugs for DAPk, especially those directed to the catalytic domain, one must consider that such drugs might affect multiple members of the death kinase family. The existence of a certain level of redundancy among the family members may in fact make this desirable.

2.2. The catalytic domain: structure and regulation

The catalytic domain of DAPk, located at the very N-terminus of the protein, consists of the typical 11 sub-domains of Ser/Thr kinases (see Fig. 1). The X-ray crystal structure of DAPk's catalytic domain has been resolved to 1.5 Å, the highest resolution for any kinase reported to date [24]. The catalytic domain has a bilobal conformation, consisting of an N-terminal domain arranged in β -sheets and a larger helical C-terminal domain, a structural feature common to many protein kinases. The ATP binding site, comprised of β -sheets, is located in the cleft formed between these two lobes (Fig. 2A). Its structure is highly similar to that of other kinases, including the positioning

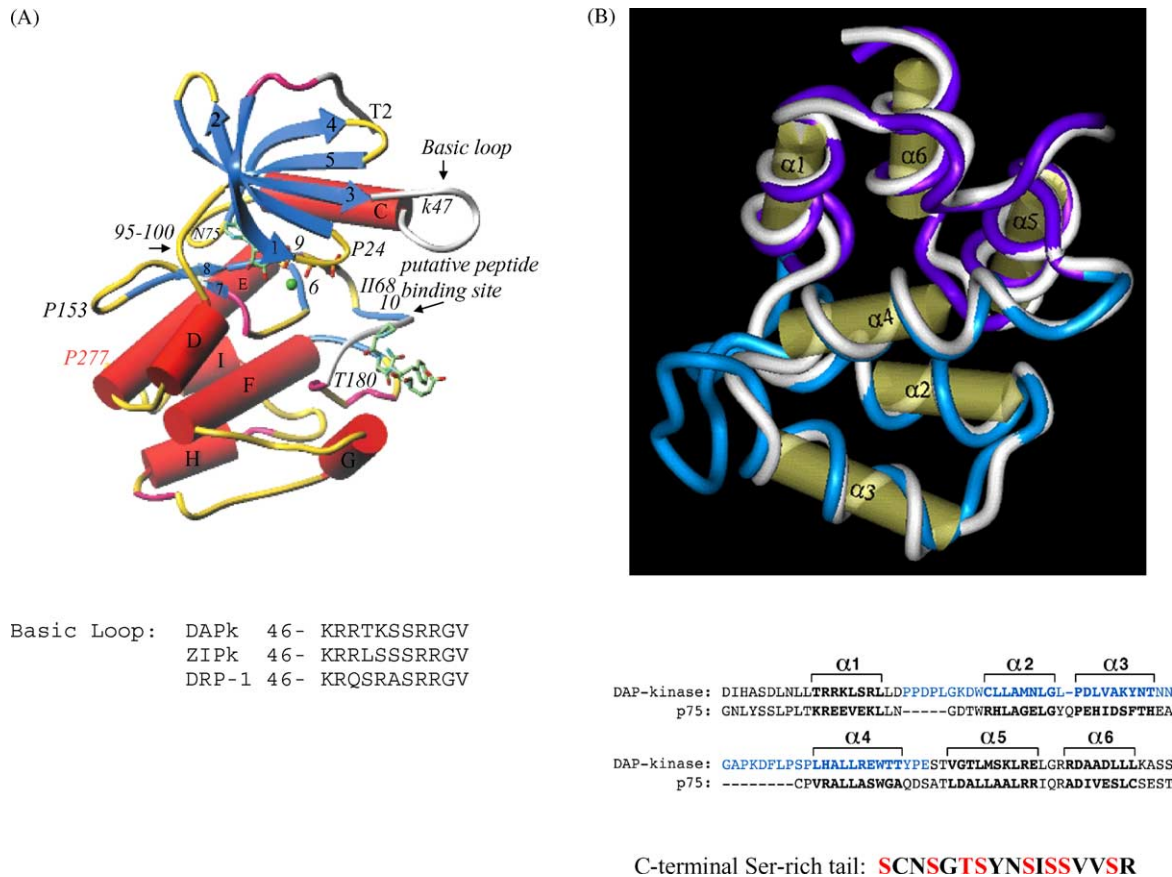
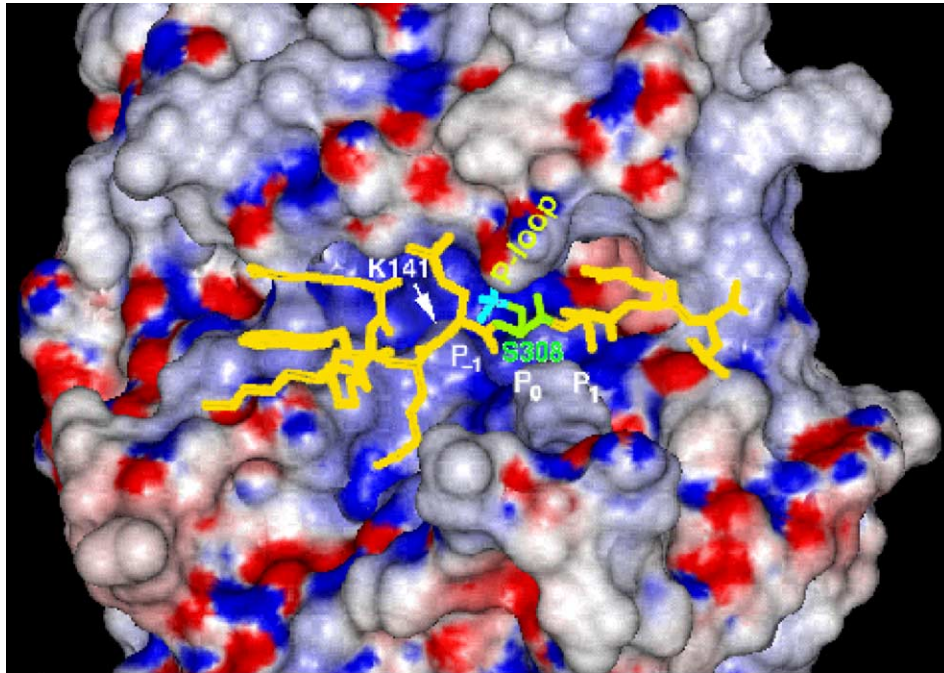


Fig. 2. (A) Ribbon diagram derived from the X-ray crystal structure of the DAPK catalytic domain. Reprinted with permission from [24]. Below the model is the sequence of the “fingerprint” of the DAPK family—the basic loop, shown for DAPk, DRP-1 and ZIP-kinase. (B) Model of DAPK’s death domain (colored ribbon), constructed by comparative modeling, overlaid onto the structure of the p75 neurotrophin receptor (white ribbon). Reprinted with permission from ref. [35]. The six α -helices are depicted in yellow. Below is the sequence alignment of the two death domains. The region from which the protective peptide was derived is shown in blue.

of Lys42, one of the amino acids critical to binding ATP [24]. In fact, DAPk loses catalytic activity upon mutation of Lys42 to Ala (K42A) [12]. In the crystal structure, the isolated catalytic domain is found in a closed, activated conformation [24]. Interestingly, sequence alignment comparisons reveal that DAPk has a unique stretch of basic residues, at amino acid position 45–57 [22]. In the crystal structure, this stretch forms a highly structured loop which protrudes from the surface of the N-terminal lobe, forming a cap-like structure over the putative peptide binding domain [24]. This sequence is highly conserved among DAPk family members, including DRP-1 and ZIP-kinase, but is not found in other CaM-regulated kinases (Fig. 2A). Thus, this loop can be considered a fingerprint of the DAPk family [24,25]. Its tight conservation within the death-associated kinases and its proximity to the substrate binding site suggest that the fingerprint loop may play an important role in substrate phosphorylation or regulation of kinase activity. Just upstream of the proposed binding region for the core substrate phosphorylation site are two clusters of acidic amino acids (E112–114, E117, and E234, E236, E238)

that may be involved in substrate binding through complementary interactions with basic residues N-terminal to the core phosphorylation site. In fact, an optimized peptide substrate contains a cluster of basic residues at the P-6 to P-8 positions, which when mutated to Ala, leads to reduced phosphorylation by 80% [26]. Thus, this acidic region of DAPk may contribute to substrate recognition, providing specificity for DAPk/substrate interactions.

Typical of the CaM kinases, the kinase domain of DAPk is followed by a CaM regulatory domain. Structural models derived from other CaM dependent kinases have led to the proposal that, at least in some cases, the CaM binding domain acts as a pseudo-substrate, interacting with the peptide binding site in the catalytic cleft, thereby blocking access to substrate [27,28]. Binding of Ca^{2+} -activated calmodulin to the CaM binding domain, however, leads to a change in conformation and a rearrangement in structure that leaves the active site exposed. It can be assumed that DAPk’s CaM-regulatory domain behaves in a similar manner, and can be computationally modeled on previously reported structural data [29] (as illustrated in Fig. 3). Yet,



CaM Regulatory Domain:

288- ASAVNMEKFKKFAARKKWKQSVRLISLCQLSR

Fig. 3. Three-dimensional model structure of the kinase domain of DAPk with a bound peptide derived from the CaM-regulatory segment. Constructed by comparative modeling as detailed in [29]. The water-accessible surface of the kinase domain is colored according to the electrostatic potential: red for negative and blue for positive potential. The peptide is shown as a stick diagram with Ser308 emphasized in green and the phosphate moiety in magenta. Below, the sequence of DAPk's CaM regulatory domain is shown; Ser308, is highlighted.

the currently solved crystal structure of DAPk does not provide any information on this critical regulatory domain. Attempts to crystallize the DAPk catalytic region with the adjacent CaM regulatory domain in the presence and absence of CaM are currently in progress. Despite the absence of structural data on the nature of this domain, however, the experimental data confirm its importance to the regulation of DAPk activity. DAPk binds calmodulin in a Ca^{2+} dependent manner *in vitro*, and is catalytically inactive in *in vitro* kinase assays in the absence of exogenous Ca^{2+} and calmodulin [12]. Deletion of the CaM binding domain from DAPk generates a constitutively active kinase (ΔCaM), which exhibits greater Ca^{2+} independent catalytic activity *in vitro* and stronger killing potential *in vivo* [12]. Furthermore, endogenous DAPk can co-immunoprecipitate with calmodulin in rat brain lysates [30]. This suggests that the CaM binding domain regulates DAPk *in vivo* as well.

In addition to binding by CaM, the CaM-regulatory domain mediates an additional level of regulation which involves auto-phosphorylation on Ser308 [29]. While auto-phosphorylation of the CaM binding domain is a common occurrence among the CaM dependent kinases [31], Ser308 phosphorylation in DAPk is unique in that it negatively regulates activity. Phosphorylation at this site is inhibited by CaM, and is inversely related to the ability of the kinase to phosphorylate exogenous substrate [29]. DAPk bearing a

mutation of Ser308 to Ala, which mimics the dephosphorylated state, has a greater affinity for CaM and is responsive to lower levels of CaM than the WT kinase. Furthermore, this mutant possesses significant CaM independent activity [29]. The converse mutation, Ser308 to Asp, which mimics the permanently phosphorylated state, results in a reduced affinity for CaM and a diminished responsiveness to CaM activation [29]. Importantly, phosphorylation regulates not only catalytic activity, but also functional activity *in vivo*. Expression of the S308A mutant leads to enhanced cell death, similar to the levels produced by constructs lacking the CaM binding domain altogether, while the S308D mutant's killing ability is greatly attenuated [29]. Most significantly, DAPk undergoes phosphorylation on Ser308 *in vivo*. This was demonstrated by immunoprecipitation of the kinase domain (amino acids 1–320) of DAPk with an anti-phosphoSer antibody. In growing 293 cells, WT DAPk, but not DAPk S308A, was successfully immunoprecipitated. However, treatment of the cells with ceramide to induce an apoptotic signal greatly reduced the immunoreactivity of the WT construct, suggesting that it had undergone dephosphorylation and was therefore no longer recognized by the anti-phosphoSer antibody [29].

Based on these results, as well as molecular modeling of DAPk's catalytic domain bound to the CaM regulatory region [29], the following model of regulation has

been proposed. In the inactive state, the CaM binding domain lies over the catalytic cleft, essentially sealing it and preventing substrate access. According to the predicted model, phospho-Ser308 lies within the catalytic cleft next to DAPk's ATP binding P-loop, with potential charge interactions between this loop, in particular Lys141, and the phosphate moiety on Ser308 [29] (see Fig. 3). These favorable interactions are predicted to stabilize the CaM binding region/catalytic cleft complex, thereby locking the molecule shut. Upon dephosphorylation, the lock is weakened due to loss of the ionic interactions between the Ser308 phosphate group and the catalytic cleft. In this state, the catalytic cleft is not tightly sealed by the CaM binding domain, and some catalytic activity towards substrate can occur even in the absence of CaM. Addition of CaM, however, completely opens the molecule, relieving the inhibition and fully activating the kinase. Furthermore, the dephosphorylated form can bind CaM to a greater extent, either due to increased accessibility or to some intrinsic increased affinity for CaM. Activation of DAPk during apoptosis would thus require some combination of two signals: increase in cellular Ca^{2+} that would enable calmodulin to bind to the CaM regulatory domain, and activation of a putative phosphatase that would dephosphorylate Ser308. It should be noted that among the DAPk family members, DRP-1 also possesses a CaM regulatory domain, and shares these modes of regulation with DAPk [32].

2.3. The extracatalytic domains

An interesting property of DAPk which has important functional implications is that the catalytic cassette comprises but a small portion of the protein (see Fig. 1). The remaining 4/5 of the protein is involved mostly in regulation and localization. Two putative P-loops of unknown function reside at positions 639–646 and 695–702, respectively. Overlapping with the second P-loop is a region that has been shown to direct the kinase to the actin cytoskeleton [12,33]. Another structural domain is found just N-terminal to the cytoskeletal interacting domain, consisting of a series of eight ankyrin repeats. The ankyrin repeats, a protein–protein interaction motif common to cytoskeletal proteins, are also necessary for the proper localization of the kinase to the actin stress fibers and for its cellular effects [33].

At the very C-terminus of the DAPk protein lies a death domain, followed by a 17 amino acid tail rich in Ser residues (SCNSGTSYNSISSVVS), a feature common to other death domain containing proteins, including death receptors and their adaptor proteins [34]. The importance of these domains to the regulation of DAPk was highlighted by an elegant study in which a complex library, comprising a random collection of DAPk cDNA fragments, was functionally screened for protein domains that were capable of inhibiting DAPk-induced apoptosis [35]. Among the random protein fragments that proved to inhibit the cellular functions of full-length DAPk were one that corresponded

to helices 2, 3, and 4 of the death domain (Fig. 2B), and one that comprised the 17 amino acid C-terminal tail.

In fact, expression of the complete death domain comprising all six helices (Fig. 2B) protected 293 cells from death by TNF- α and Fas, and Hep3B cells from TGF β -induced apoptosis [13,16]. Deletion of the death domain attenuates DAPk's killing ability, indicating that it is necessary for DAPk function, perhaps by interacting with some critical activator or substrate [13,36]. The most likely explanation for its inhibitory nature *in trans* is that the death domain fragment competes with the full length kinase by sequestering this putative death-domain interacting partner. The identity of this interacting partner is currently unknown yet under extensive study in several laboratories. Moreover, there is no indication that the DAPk death domain can homodimerize or interact with any of the well known death domains, including that of Fas [13].

Studies on the C-terminal tail revealed an opposite intrinsic property of this amino acid stretch. Although the C-terminal tail fragment can also inhibit the death promoting effects of DAPk *in trans* when co-introduced into cells, in contrast to the death domain, it has an auto-inhibitory function [35]. Deletion of the last 17 amino acids from the protein activated the death-promoting effects of DAPk. As the tail deletant did not exhibit any difference in *in vitro* kinase activity when compared to the wild type [35], the tail's mode of action must exist at a level other than catalytic activity. One possibility would be that it folds over some other functional module of DAPk, such as the ankyrin repeats or the death domain, and thus reduces its cellular function without affecting directly the catalytic activity.

In conclusion, two distinct auto-inhibitory mechanisms which are relevant to the death-promoting effects of the kinase were discovered by structural dissection of the protein. One of these is mediated by an inhibitory type of autophosphorylation occurring on a single site within the CaM regulatory domain. Changes in Ca^{2+} /CaM concentrations concomitant with changes in the status of this autophosphorylation control the catalytic activity, which is critical for the cellular effects. The second auto-inhibitory mechanism relies on the presence of the 17 amino acid C-terminal tail. Although the tail's molecular mode of action is less clear at the present time, it can provide a basis for specific drug development, as detailed below.

3. Mode of action of DAPk in cell death

Over the last few years, several groups have joined the effort to decipher DAPk's cellular function, focusing on areas that include its biochemical properties, regulation and target substrates. From these studies, it has become apparent that DAPk has multiple functions and that it lies at a critical executive juncture of cell death signaling. Once activated, it can trigger a range of death responses leading to multiple phenotypes. All phenotypes elicited require catalytic

activity (e.g. [12,16,22,37]), indicating that each functional arm of DAPk results from phosphorylation of a particular substrate or set of substrates, although some substrates may be shared by more than one functional arm.

In certain cell types, expression of DAPk leads to the appearance of autophagic vesicles, membrane blebbing, and extensive cell rounding, features characteristic of an alternate type of programmed cell death, referred to as type II or autophagic cell death [17]. This occurs without apparent caspase activation, and represents a caspase-independent cell death pathway. Furthermore, DAPk is necessary for autophagosome formation in cell culture models of autophagic cell death, such as IFN- γ -mediated killing of HeLa cells [17]. Autophagy, a process in which double-membrane vesicles develop and consume intracellular contents, is often utilized by the cell as a maintenance mechanism to clean up damaged organelles. In times of stress, especially, it is used to generate an emergency supply of essential nutrients through the targeted breakdown of cellular components. Above a certain threshold, however, autophagy can be cytotoxic, leading to the excessive consumption of organelles and the eventual death of the cell [10]. As a regulator of autophagy, DAPk may be critical for stress-related disorders in which this mode of cell death predominates.

While the specific substrates of DAPk that mediate autophagosome formation are not known, the mechanism leading to membrane blebbing is better understood. DAPk, situated at the actin stress fibers and cortical actin ring [33], phosphorylates the regulatory light chain of myosin II (MLC), both *in vitro* and *in vivo*, on Ser19 [12,33,36,38]. Phosphorylation of MLC in this manner activates myosin contractility, an event critical for the formation of membrane blebs [39]. DAPk is not only sufficient to induce membrane blebbing during type II cell death, but is also necessary for the full extent of blebbing that accompanies caspase-dependent TNF α -induced apoptosis [17]. The increased cell contractility that results from DAPk-mediated activation of myosin might also contribute to the rounding of the cell that is typically observed upon DAPk expression. Furthermore, DAPk has been shown to affect the adhesion of cells to extracellular matrix by interfering with integrin signaling [37]. In anchorage-dependent growth conditions, loss of adhesion in certain cell types can subsequently activate a caspase-dependent cell death process known as anoikis [37].

In some circumstances, DAPk has been linked to the typical caspase-dependent apoptosis signaling pathway. For example, in the presence of active p53, DAPk mediates caspase-dependent apoptosis in response to hyperproliferative signals in primary fibroblasts [15]. Caspase-dependent apoptosis is similarly activated by DAPk during TGF β -killing of hepatoma cells [16]. Thus, while on its own, DAPk will trigger caspase-independent death processes, in the proper signaling milieu, its activity can be recruited to mediate other death pathways. The choice of pathways elicited by activated DAPk will depend

on the cell context. An important initial determining factor in the overall phenotype will be the substrates which the kinase encounters. But ultimately, the final outcome after substrate phosphorylation will be determined by the presence or abundance of factors that interact with or modulate these substrates and the signaling pathways that they regulate.

4. DAPk as a drug target in neuronal cell death

Currently, there is no approved drug that protects the brain from the damage that results from a stroke or traumatic brain insult. Following a stroke or brain injury, neurons continue to die over an interval lasting from hours to days, adding significantly to the initial damage. This secondary cell death would be the most likely target for intervention. Novel drugs that target neuronal apoptotic regulators would potentially block the destructive chain reaction that occurs in the brain hours after stroke or traumatic brain injury. DAPk has created considerable interest in the pharmaceutical industry as a new target for the development of such drugs. This section will present the evidence supporting a role for DAPk in mediating cell death of the central nervous system (CNS), and describe initial efforts in the development and application of DAPk specific inhibitors.

DAPk, although ubiquitously expressed in almost all adult mouse tissues, is particularly abundant in the brain (Raveh et al., unpublished data). During embryonic development, DAPk mRNA is found at high levels in the brain, especially in the cerebral cortex, cerebellar Purkinje cells, and hippocampus [40]. After birth, DAPk mRNA is restricted to the hippocampus. At the protein level, DAPk is limited to the cortex, hippocampus and olfactory bulb in the adult rat brain [41]. Presumably, the high levels of DAPk observed in the CNS are tightly regulated in the basal state so as not to induce premature cell death.

Data from several labs indicates that DAPk can be specifically activated in neurons following death stimuli. For example, a rapid, transient increase in both CaM dependent and independent catalytic activity of endogenous DAPk was observed following ceramide treatment of PC12 cells [18]. The significance of this correlation was emphasized by the observation that over-expression of DAPk enhanced the apoptotic response of PC12 cells to C₂- and C₈-ceramide [18]. Additionally, increases in DAPk catalytic activity were observed in homogenates isolated from injured hippocampal tissue following neonatal cerebral hypoxia-ischemia [42]. In some cases, DAPk regulation occurred at the level of expression. DAPk levels increased three-fold in the cerebral cortex 24 h following transient forebrain ischemia [40]. Likewise, protein increases were observed upon treatment of cultured hippocampal neurons with C₆-ceramide [19]. Seizure-induced neuronal death in the rat hippocampus and cortex was also accompanied by increased DAPk immunoreactivity, specifically in the affected regions [30].

Several experiments, performed either in animal models, including DAPk knock out mice, or in primary neuronal cell cultures derived from normal and deficient mice, establish DAPk's necessity for neuronal cell death. In cell culture, primary hippocampal neurons which lack DAPk were much more resistant than their WT counterparts to ceramide-mediated death signals, triggered either directly by administration of C₆-ceramide or indirectly by activation of p75 neurotrophin receptors with NGF [19]. Likewise, dominant negative mutants of DAPk rescued PC12 cells from C₂ and C₈-ceramide induced apoptosis [18]. In vivo, retinal ganglion cells from DAPk^{-/-} mice showed increased survival (79% versus 56% in matched controls) following administration of glutamate [43]. This is particularly significant, as cytotoxic levels of extracellular glutamate accompany, and are thought to contribute to, numerous neurodegenerative disorders, such as cerebral ischemia, brain injury and amyotrophic lateral sclerosis [44,45].

DAPk's prominent role in neuronal cell death, combined with the fact that as an enzyme, it presents a particularly attractive target for inhibition, initiated two independent approaches to drug development. The first approach took advantage of the inhibitory nature of the tail of DAPk [35]. A peptide corresponding to the 17-amino acid tail region of DAPk was synthesized and covalently linked at its N-terminus to tetramethylrhodamine isothiocyanate, in order to increase the hydrophobicity of the peptide, and to enable its detection upon uptake into cells [19]. Significantly, administration of micromolar concentrations of the labeled peptide to the culture media partially protected primary hippocampal neurons from cell death induced by C₆-ceramide or expression of sphingomyelinase [19]. In fact, the reduced sensitivity of hippocampal neurons to various death signals perfectly resembled the behavior of DAPk deficient neurons. This encouraging data suggests that the tail peptide can act as a starting point for optimization of a peptide-based therapy, to generate a specific inhibitor of DAPk that is both more stable and more practical for in vivo delivery.

The second approach used DAPk's crystal structure to design catalytic site chemical inhibitors of DAPk [46]. An alkylated 3-amino-6-phenylpyridazine was developed, which inhibited DAPk's catalytic activity with an IC₅₀ of 13 μM. The IC₅₀ was more than 10-fold lower than that observed when CaMKII, PKA or PKC were tested with this compound, indicating that the compound showed some degree of specificity. Its activity towards other related kinases such as MLCK, or the DAPk homologues DRP-1 and ZIP-kinase was not evaluated, however. The co-crystal structure of the inhibitor bound to DAPk's catalytic domain was determined, and revealed that the drug partially occupied the ATP binding site, with the inhibitor's phenyl ring located in a similar position to ATP's adenine ring [46]. The proof of concept that inhibition of DAPk with this small molecule can attenuate pathologies of the CNS was demonstrated in a rat model of hypoxia-ischemia. A single intraperitoneal administration of this inhibitor just prior to ligation of the

carotid artery resulted in a significant decrease in brain tissue loss (17% reduction in brain hemisphere weight versus 30% loss in control-treated rats) measured 1 week following injury [46]. Significantly, this protection was maintained even upon delivery of the compound 6 h following injury, a more realistic time-scale for therapeutic intervention in a clinical setting [46]. Thus, this aminopyridazine molecule is a very promising lead compound for further drug development of a specific, deliverable small molecule inhibitor of DAPk.

5. Clinical implications in cancer: DAPk as a diagnostic/prognostic factor and as a target for tumor treatment

Obviously, one of the challenges in the study of DAPk is to find out whether this pro-apoptotic gene is a potential tumor suppressor subjected to loss or inactivation in cancer. To this end, several independent directions were undertaken in the past several years, including experimental investigations into the effects of loss of DAPk on tumorigenesis, as well as extensive screens of human tumors. The experimental approaches, performed both in cell cultures and in mouse model systems, established that DAPk functions as a tumor suppressor in at least two different stages of tumorigenicity, namely, an apoptotic checkpoint functioning early during cell transformation, and a second one that occurs later in cancer development, i.e., during metastasis. In parallel, a comprehensive screen of human tumor specimens was conducted and correlations were made with respect to the staging and aggressiveness of the disease in various types of cancer. In fact, in the past 3 years alone, over 100 articles have been published on the status of DAPk expression in human tumors as summarized in detail in the next sections.

5.1. DAPk functions as a tumor suppressor in cell culture and animal models

Cell-based experiments performed in primary mouse embryonic fibroblasts (MEFs) suggested a role for DAPk in an apoptotic checkpoint which is turned on by oncogenes early during cell transformation. Briefly, these experiments showed that DAPk is necessary for the full apoptotic response to oncogenic transformation detected in wild type MEFs. Fibroblasts derived from DAPk^{-/-} mice showed decreased induction of p53 and p19^{ARF}, and consequently, decreased levels of apoptosis following the hyperproliferative signals generated by forced expression of oncogenes such as c-myc and E2F [15]. Based on these data, it was suggested that DAPk serves as a safeguard mechanism against early steps in cancer development (for more details, the reader is referred to a previous review [47]).

It had been observed in our laboratory that highly metastatic clones originating from Lewis lung cell carcinoma were DAPk negative, whereas the low metastatic counterparts of these cells expressed normal DAP-kinase

levels. Based on this finding, an animal model system was employed to test the involvement of DAPk in tumorigenesis *in vivo*. Metastatic activity was examined by intravenous injection of these carcinoma cells into syngeneic mice. Strikingly, restoration of physiological levels of DAPk into the originally highly metastatic cells suppressed their ability to form lung metastases in mice. Conversely, the rare lung lesions which spontaneously arose in the mice following injection of the original poorly metastatic cells had lost endogenous DAPk expression at high frequency. Further assessment of the apoptotic index of these tumors indicated that expression of the DAPk transgene re-sensitized the tumor cells to apoptotic stimuli, a property which they had originally managed to circumvent by losing DAPk. Based on this, it has been suggested that DAPk-mediated suppression of metastasis results, at least in part, from increased sensitivity to various death-inducing stimuli [14]. It is also conceivable that DAPk's cytoskeletal functions, such as its effects on adhesion and cell contractility, might influence various stages of metastasis, such as tumor migration and invasion. Thus, loss of DAPk expression provides a positive selective advantage during the formation of lung metastases.

5.2. Screens of human tumors: promoter methylation as a means to silence DAPk in cancer

Consistent with the experimental evidence for DAPk's tumor suppressor function, many human tumor cells, including cell lines derived from tumors as well as biopsies of human tumors, were found to lack DAPk expression. The tumor cell lines that have a high incidence of loss of DAPk expression include those derived from B and T cell malignancies [48–50], colon [51], nasopharyngeal [52], ovarian/uterine [53], oral [54], lung [55] and colorectal cancer [56]. We and others found that treatment of these tumor cell lines with 5-aza-2'-deoxycytidine led to demethylation and re-expression of DAPk in most, yet not all cases [48,50,51,54–56]. This indicated that aberrant DNA methylation is one of the mechanisms responsible for silencing this gene. Indeed, a CpG island at the 5'UTR of DAP-kinase was found to be a target for hypermethylation by Herman and his co-workers [57]. The biological relevance for this hypermethylation was first demonstrated in the Raji Burkitt's lymphoma cell line, in which DAP-kinase is fully methylated and not expressed. Demethylation of the DAPk gene by treatment with 5-aza-2'-deoxycytidine restored not only the DAPk expression, but also, significantly, the cells' apoptotic sensitivity to IFN- γ [57]. Not surprisingly, as with many other tumor suppressor genes, homozygous gene deletion and loss of heterozygosity of DAPk have also been observed, although less frequently than hypermethylation ([58]; Kissil and Kimchi, unpublished observations) (see also Fig. 4B)]. Conceivably, tumors which fail to show methylation of the DAPk gene may experience other genetic events that act to functionally inactivate DAPk. Clearly,

anti-neoplastic therapies must take into account both DAPk expression and function.

Gene silencing events that involve transcriptional inactivation associated with abnormally methylated promoter CpG islands is a fundamental feature of human cancer. Using methylation-specific polymerase chain reaction (MSP) assays developed by Herman and his co-workers, the methylation status of DAP-kinase's 5'UTR was extensively analyzed in DNA extracted from normal and tumor samples by many groups and in various types of carcinomas, sarcomas and lymphomas. The MSP method is based on the resistance of methylated cytosines to chemical modification by sodium bisulfide treatment and on the subsequent use of specific primers which correspond to the identified methylated region containing the CpG island. A large variety of human primary tumor biopsies was screened for the status of DAPk expression by this method (an example performed in our laboratory is illustrated in Fig. 4A). While in some cases, in particular pediatric tumors, DAPk gene methylation was low or undetected [57,59–61], the vast majority of tumors types had significant levels of methylation as compared to normal tissue counterparts. The highest frequency of DAP-kinase methylation was detected in B-cell lymphoma. All Burkitt's lymphoma tested and 84% of B-cell non-Hodgkin's lymphomas were hypermethylated in the DAP-kinase CpG island [57].

One theme that emerges from these tumor screens is that the high incidence of DAPk methylation provides a new means for diagnostic screening. More importantly, however, is data that indicates that loss of DAPk may play a causative role in tumor development. Consistent with this, loss of DAPk is observed in some pre-malignant stages of gastric carcinoma [59,62], and in the normal mucosa adjacent to oral squamous cell carcinomas of tobacco-chewing patients [63]. In other studies, a correlation between DAPk methylation status and tumor severity, increased disease recurrence and metastasis, and decreased survival rates, has been demonstrated. Loss of DAPk was associated with metastasis in pituitary tumors [58], and head and neck cancer [64,65]. Likewise, increased tumor size, pathologic state and lymph node involvement correlated with DAPk loss in non-small cell lung cancer [66]. DAPk methylation occurred at a higher rate in more advanced grades and stages of uterine cervical carcinomas [51,67], astrocyte glioma [68] and head and neck cancer [64]. Survival rates were reduced three-fold in patients suffering from multiple myelomas that exhibited DAPk methylation [69], and an 88% recurrence rate was observed in superficial bladder cancer patients in which DAPk was methylated, compared to 28% of non-methylated patients [70]. Furthermore, DAPk expressing hepatocellular carcinomas showed a higher tumor differentiation rate, fewer metastatic foci and decreased portal invasion, a greater apoptotic index, and, ultimately, increased survival rate compared to tumors that lacked DAPk expression [71]. However, in other studies, including urinary bladder cancer [72], nasopharyngeal carcinoma [73], esophageal adenocarcinoma

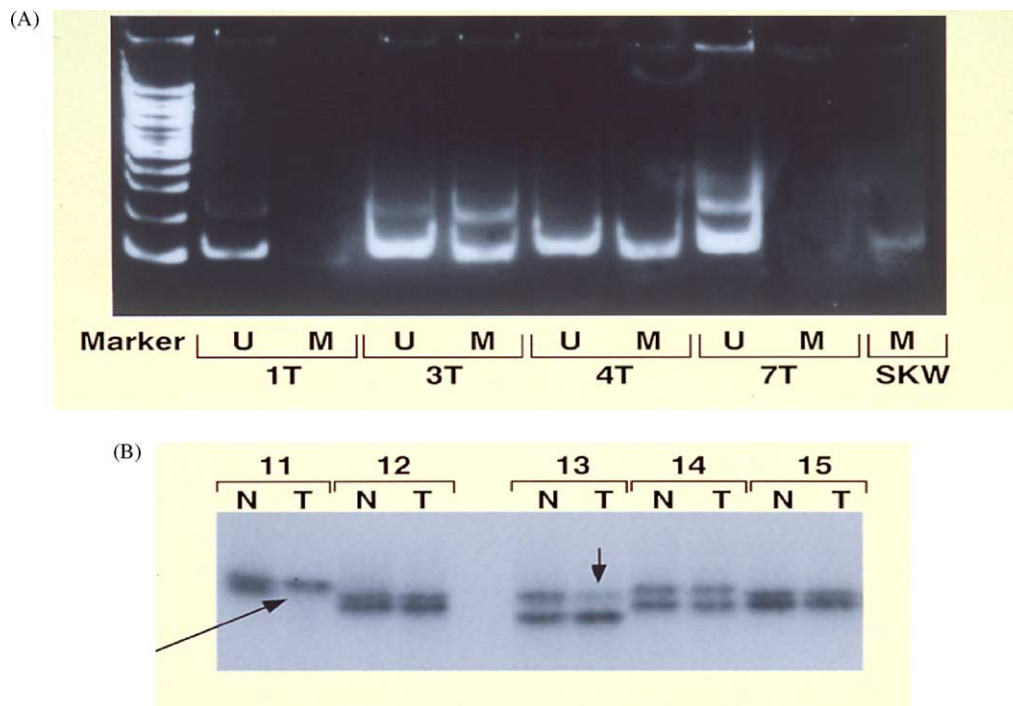


Fig. 4. Epigenetic and genetic changes in the DAPk gene in tumors. (A) Example of a screen of human tumor specimens by methylation specific-PCR. U-DNA fragments amplified by primers corresponding to unmethylated CpG island; M-DNA fragments amplified by primers corresponding to methylated CpG island. T-tumor annotation. (B) Example of loss of heterozygosity of the DAPk gene (marked by arrows) in tumors (T), compared to normal tissues (N) from the same patient.

[74], invasive lobular breast cancer [75], non-small cell lung cancer [76], colorectal cancer [77], and gastric cancer and intestinal metaplasia [62], DAPk promoter methylation was detected without any association with the stage of disease.

Of note, DAPk is usually not the only gene whose expression is lost; several tumor suppressor genes are known to be affected by DNA methylation including, for example, p16 INK4a, p15INK4b, p14ARF, Von Hippel-Lindau (VHL), APC, and E-cadherin [78–80]. Indeed, many studies have focused on analysis of multi-gene methylation, including DAPk (e.g. [63,81]). Although the incidence of DAPk methylation is high in tumor samples, tumor development and progression may be a result of the simultaneous loss of multiple genes. For example, in one analysis of pediatric ALL, DAPk methylation status correlated with methylation of p16INK4A [82]. Harden et al., found that methylation of both APC and DAPk was associated with a more severe outcome; death was the outcome in 54% of patients with methylation in both genes compared with 27% when only one was methylated [83].

Aberrant DNA hypermethylation has now become a major focus for developmental therapeutics in cancer [79,80]. As methylation is a reversible process, agents capable of demethylating DNA and re-activating silenced genes are ideal candidates for cancer therapy. Such drugs include 5-azacytidine and 5-aza-2-deoxycytidine (Decitabine), which are approved drugs already in clinical use. Despite their promise, however, Decitabine and 5-azacytidine are

chemically unstable and neither can be given orally, thus limiting their clinical use. A new demethylating agent, 1-(β -ribofuranosyl)-1,2-dihydropyrimidine-2-one (Zebularine), a chemically stable cytidine analogue, has been shown to significantly reduce bladder carcinoma tumor volume in BALB/c nu/nu mice and to reactivate the p16INKA gene in these cells [84]. Significantly, Zebularine is the first orally active drug that can re-activate epigenetically silenced genes. An important drawback of the demethylating agents currently available is that they cannot be targeted to specific genes, but rather globally demethylate many genes. One must thus consider possible long-term (and unknown) effects of reactivating genes that are normally not expressed.

6. Perspectives and prospects for cancer treatment

The extensive promoter methylation screens combined with the experimental data have established the tumor suppressor functions of DAPk and highlighted its importance in the diagnosis/prognosis of cancer. In the future, DAPk-based therapeutic modalities should be designed, taking into consideration the general progression of gene therapy. For example, as the clinical feasibility of gene therapy increases, activated forms of DAPk, such as Δ CaM or Δ tail DAPk mutants, can be used to eliminate the target cells through the death-promoting functions of the activated kinase. The high abundance of promoter silencing by hypermethylation

opens the road to the use of demethylating agents, the limitations of which were discussed above. Only once the molecular mechanisms which control the methylation process are thoroughly dissected, including identification of the specific factors that direct the multi-protein methylation complex to specific gene promoters, can one design more specific methods of targeting the desired gene.

Another future therapeutic strategy may be directed to those tumors in which DNA methylation did not completely eliminate DAPk expression levels [47]. In these cases, clever strategies aimed at activating the residual protein levels may be effective. One potential direction would be to develop methods of interfering with DAPk's auto-inhibitory mechanisms. For example, low molecular weight compounds that block the binding of Ser 308 of the CaM regulatory domain to the catalytic cleft might release the kinase from inhibition by this domain, thus activating the kinase. Alternatively, agents that antagonize the C-terminal tail's self inhibitory activity can also be pursued. Obviously, these approaches are still in their hypothetical stages, and their future development will depend on having the detailed X-ray crystal structure of these domains in their inhibitory states.

Finally, as in every gene-based therapeutic modality, the combinatorial nature of tumor progression should be considered. The status of other tumor suppressor genes and oncogenes in a given tumor will influence the impact of DAPk activation. Identifying the different molecular pathways which lead to cell death and their integration with the DAPk-pathways will dictate which combination of genes should be targeted simultaneously in order to achieve the most efficient elimination of the cancer clone in each individual tumor.

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