

Review

# The DAP-kinase family of proteins: study of a novel group of calcium-regulated death-promoting kinases

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## Abstract

DAP-kinase (DAPk) is a  $\text{Ca}^{2+}$ /calmodulin (CaM)-regulated Ser/Thr kinase that functions as a positive mediator of programmed cell death. It associates with actin microfilament and has a unique multidomain structure. One of the substrates of DAPk was identified as myosin light chain (MLC), the phosphorylation of which mediates membrane blebbing. Four additional kinases have been identified based on the high homology of their catalytic domain to that of DAPk. Yet, they differ in the structure of their extracatalytic domains and in their intracellular localization. One member of this family, DRP-1, also shares with DAPk both the property of activation by  $\text{Ca}^{2+}$ /CaM and a specific phosphorylation-based regulatory mechanism. The latter involves an inhibitory type of autophosphorylation on a conserved serine at position 308, in the CaM regulatory domains of these two kinases. This phosphorylation, which occurs in growing cells, restrains the death-promoting effects of these kinases, and is specifically removed upon exposure of cells to various apoptotic stimuli. The dephosphorylation at this site increases the binding and sensitivity of each of these two kinases to their common activator—CaM. In DAPk, the dephosphorylation of serine 308 also increases the  $\text{Ca}^{2+}$ /CaM-independent substrate phosphorylation. In DRP-1, it also promotes the formation of homodimers necessary for its full activity. These results are consistent with a molecular model in which phosphorylation on serine 308 stabilizes a locked conformation of the CaM regulatory domain within the catalytic cleft and simultaneously also interferes with CaM binding. In DRP-1, it introduces an additional locking device by preventing homodimerization. We propose that this unique mechanism of autoinhibition, evolved to keep these death-promoting kinases silent in healthy cells and ensures their activation only in response to apoptotic signals.

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## 1. Introduction

Apoptosis (programmed cell death) is a genetically controlled cell death process, which is important at various developmental stages, as well as in the maintenance of tissue homeostasis [1]. During the past few years, many of the key players in this process have been identified, including receptors, adapter proteins, proteases and other positive and negative regulators. One of the positive mediators of apoptosis, which was cloned in our laboratory, is DAP-kinase (DAPk) [2]. DAPk was discovered by a functional approach to gene cloning, based on transfection of mammalian cells with antisense cDNA library and subsequent isolation of death protective cDNA fragments [2,3]. Anti-

sense DAPk RNA expression protected HeLa cells from interferon- $\gamma$ -induced cell death. DAPk is a pro-apoptotic Ser/Thr kinase that participates in a wide spectrum of apoptotic signals including IFN- $\gamma$ , TNF- $\alpha$ , activated c-Myc, TGF- $\beta$  and detachment from extracellular matrix [2,4–7]. This actin microfilament-associated  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent kinase has a unique multidomain structure including modules that mediate protein–protein interactions such as ankyrin repeats and the death domain (Fig. 1). The death-promoting effects of DAPk depend on its catalytic activity, the correct intracellular localization, and on the presence of the death domain [4].

Four additional kinases that show a significant homology in their catalytic domain to DAPk have been recently identified [8]. ZIP(Dlk)-kinase and DRP-1 are the closest family members, as their catalytic domains share approximately 80% identity to that of DAPk. Two more distant DAPk-related proteins are DRAK1 and DRAK2 (Fig. 2).

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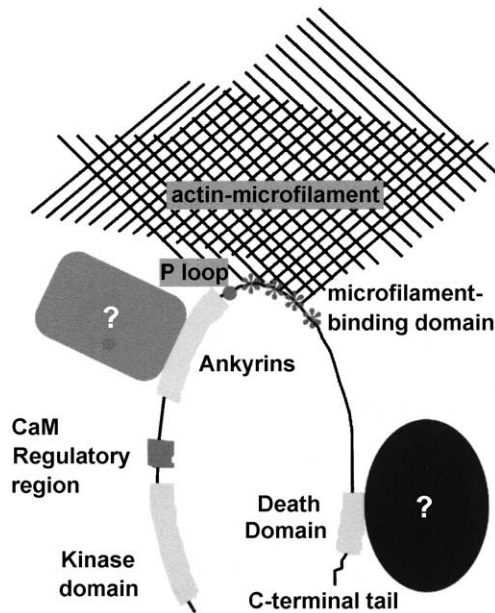


Fig. 1. A scheme showing the multidomain structure of DAPk and the position of the region that is required for the actin microfilament binding. The question marks refer to yet unidentified proteins that may bind to the death domain and to the ankyrin repeats.

Phylogenetic analyses, based on multiple sequence alignment of the catalytic domains of 16 proteins, show that DAPk, ZIP(Dlk)-kinase and DRP-1 may be grouped into a distinct clade with high bootstrap probabilities. DRAK1 and DRAK2 form another clade sharing a putative common ancestor to the other DAPk related proteins. Interestingly, the extracatalytic domains of these five members differ considerably from each other (Fig. 2). Additionally, ZIP-kinase, DRAK1 and DRAK2 were shown to be nuclear proteins that do not require  $\text{Ca}^{2+}$ /CaM for activation. DRP-1, however, is a cytoplasmic kinase, containing a typical CaM-regulatory domain similar to that of DAPk and a short

C-terminal segment required for homodimerization. [8,9]. Interestingly, DRP-1 can promote cell death upon its ectopic expression and it shares with DAPk many of the induced subcellular events including membrane blebbing and the formation of autophagic vesicles [10]. This paper summarizes the current information available with respect to the mode of activation of these two closely related kinases during cell death.

## 2. DAPk mode of activation during programmed cell death

The apoptotic function of DAPk must be under tight control, to ensure on one hand its silence under normal growth conditions, and to allow, on the other hand, rapid activation in response to the appropriate apoptotic signal. While extensive work was invested in characterizing the apoptotic systems where DAPk functions, with respect to the structure/function features of the protein, and to the impact of its loss in cancer development [2,4–6,10–13], the mechanism of DAPk activation has only recently been deciphered in detail.

The identification of myosin light chain (MLC) as a substrate of DAPk facilitated the performance of *in vitro* kinase assays enabling the analysis of different aspects of its catalytic activity and of its mode of regulation. Structure/function dissection of the protein revealed two molecular layers of regulation, which control the catalytic activity and hence the pro-apoptotic effect of DAPk. The first comprises the CaM-regulatory segment, which possesses an autoinhibitory effect on the catalytic activity, relieved by binding to  $\text{Ca}^{2+}$ -activated CaM [4]. Consistently, the mere deletion of this segment from DAPk ( $\Delta\text{CaM}$  mutant) generated a constitutively active kinase, which displayed CaM-independent substrate phosphorylation *in vitro* and promoted the apoptotic activity *in vivo* [4]. In addition to the basic regulatory mechanism of CaM-mediated relief from auto-

### The DAP-kinase family of proteins

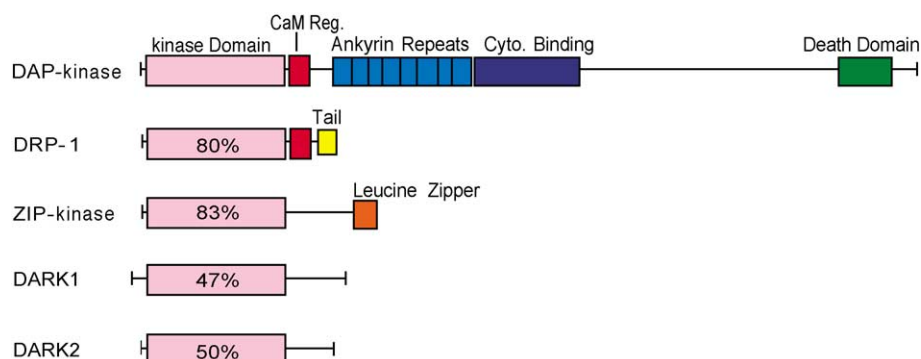


Fig. 2. DAPk family members. The percent identity among the catalytic domains is indicated and different structural modules are shown.

inhibition, a second layer of regulation that is mediated by an autophosphorylation mechanism was recently revealed [14].

The relevant autophosphorylation site was mapped to Ser<sup>308</sup> which resides within the CaM regulatory segment of the enzyme. In vitro kinase assay revealed that the autophosphorylation of Ser<sup>308</sup> is Ca<sup>2+</sup>/CaM-independent and is strongly inhibited by the addition of Ca<sup>2+</sup>/CaM. The biochemical implication and functional relevance of the phosphorylation status of Ser<sup>308</sup> was then established by two single-point mutations mimicking the different phosphorylation forms of this specific site. DAPk in which Ser<sup>308</sup> was converted to alanine served to simulate a dephosphorylated state of Ser<sup>308</sup>, while the substitution to aspartic acid mimicked the phosphorylated form of Ser<sup>308</sup>.

Biochemical analysis of these mutants revealed two distinct properties which are influenced by the phosphorylation status of serine 308. The first comprised an in vitro elevation in the basal Ca<sup>2+</sup>/CaM-independent catalytic activity upon Ser to Ala substitution. This Ca<sup>2+</sup>/CaM-independent activity was minimized by the Ser to Asp substitution [14]. It is suggested based on these data that Ser<sup>308</sup> autophosphorylation strengthens a “locking device”, probably generated by the interaction of the CaM-autoinhibitory domain with the catalytic cleft. Absence of the phosphate group, conversely, weakens this “lock” and therefore partially relieves the autoinhibition, resulting in Ca<sup>2+</sup>/CaM-independent activity. The second biochemical property, which is governed by Ser<sup>308</sup> phosphorylation status, is the CaM binding activity. The prevention of Ser<sup>308</sup> phosphorylation resulted in a marked elevation in CaM binding activity as assessed by two different CaM binding assays in vitro [14]. Conversely, the opposing substitution to aspartic acid strongly reduced the binding to CaM. The elevation could result from enhanced accessibility of the dephosphorylated form to interaction with CaM due to the “weakened lock”, and/or from an intrinsic property of the dephosphorylated segment that permits a better interaction with CaM. Thus, different aspects of the catalytic activity of DAPK can be affected by the status of Ser<sup>308</sup> phosphorylation.

Next, it became of interest to test whether DAPk is subjected to dephosphorylation in response to an apoptotic stimulus in cells. We started by verifying the existence of Ser<sup>308</sup> autophosphorylation in vivo and demonstrated by using anti-phosphoserine antibodies that indeed DAPk undergoes autophosphorylation on Ser<sup>308</sup> in growing cells. Of importance was the finding that Ser<sup>308</sup> undergoes dephosphorylation in response to ceramide which utilizes active DAPk as part of its mode of action in some cells [14].

The cellular relevance of these two phosphorylation forms of DAPk in vivo was studied by analyzing the cell death-promoting capacity of the single mutants in transfection-based assays. The S308D substitution, mimicking phosphorylation and simulating the state of DAPk in grow-

ing cells, gave rise to a ‘loss of function’ mutant which possesses very minor killing activity. In contrast, S308A representing imposed dephosphorylation, and simulating the state of DAPk in response to an apoptotic stimulus, generated a ‘super-killer’ mutant. Thus, phosphorylation on Ser<sup>308</sup> silences the pro-apoptotic activity, whereas dephosphorylation of Ser<sup>308</sup> is part of the activation process of DAPk [14].

### 3. DRP-1 mode of activation during programmed cell death—the ‘double locking’ mechanism

DRP-1, also named DAPk-2, was cloned based on its homology to the DAPk catalytic domain. As in the case of DAPk, in vitro kinase assays confirmed the ability of DRP-1 to phosphorylate an exogenous substrate, MLC, in a Ca<sup>2+</sup>/CaM-dependent manner. Removal of the CaM-regulatory domain converted the enzyme into a constitutively active, Ca<sup>2+</sup>/CaM-independent form. Overexpression of DRP-1 induced cell death, and a dominant negative DRP-1 mutant protected cells from TNF- $\alpha$ -induced apoptosis, indicating the involvement of DRP-1 in apoptosis [8]. It was found that the death-promoting effects of DRP-1 depend on the integrity of its catalytic activity, as a catalytic inactive DRP-1 mutant was incapable of inducing apoptosis. Most importantly, the last 40 amino acids comprising the carboxy-terminal tail, which show no homology to DAPk or to any other known proteins, were found to be required for DRP-1 homodimerization. Interestingly, the death-promoting effects of DRP-1 depended on the presence of this C-terminal tail. Yet, in the absence of the CaM-regulatory domain, the C-terminal tail became dispensable in these death assays, an enigma which could not be resolved previously due to the lack of data concerning the precise mode of DRP-1 regulation.

Obviously, one of the most challenging issues concerning DRP-1 study related to its mode of activation during programmed cell death. The starting point in this respect was the seminal finding that DRP-1 undergoes massive autophosphorylation at basal state in the absence of Ca<sup>2+</sup>/CaM and that an inverse relationship exists between this autophosphorylation and the MLC substrate phosphorylation in a sense that Ca<sup>2+</sup>/CaM stimulated the latter and strongly suppressed the former [9]. It was found that this autophosphorylation comprises a single phosphorylation event that was mapped by point mutations and mass spectrometry to Ser<sup>308</sup> within the CaM-regulatory domain. Thus, the high degree of conservation among the CaM-regulatory domains of DAPk and DRP-1 (Fig. 3) is also reflected by a common phosphorylation site whose functional relevance in the context of DRP-1 was then studied in details.

A mutation that mimicked phosphorylation on Ser<sup>308</sup> (i.e., substitution to aspartic acid) abrogated the ability of DRP-1 to induce apoptosis, while the reciprocal serine to

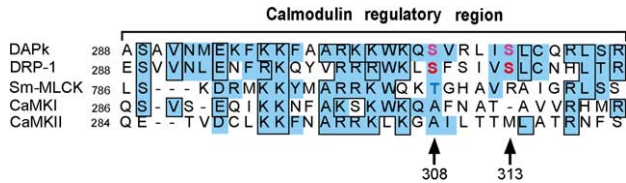


Fig. 3. Multiple sequence alignment of CaM regulatory domains of DAPk, DRP-1, smMLCK, CaMKI and CaMKII. Identical amino acids are boxed; homologous amino acids are shown in blue. Arrowheads point to candidate phosphorylation sites in the CaM regulatory domains of DRP-1 and DAPk, suggested in light of their exclusive conservation in the two kinases. Among these two residues, Ser<sup>308</sup> was experimentally found to be the major site for autophosphorylation in contrast to Ser<sup>313</sup> which was hardly phosphorylated. Mutations in this site had no functional implications [9,14].

alanine substitution gave rise to a “super-killer” mutant. This further proved the biological importance of this single negative autophosphorylation, which restrains the apoptotic features of the protein. At the biochemical level, the substitution of Ser<sup>308</sup> to aspartic acid decreased the binding affinity to CaM leading to reduced substrate phosphorylation at limiting CaM concentrations. The reduced CaM binding was assessed either directly by measuring the binding of <sup>35</sup>S-labelled CaM to the recombinant proteins or indirectly by measuring MLC phosphorylation under rate limiting CaM concentrations [9]. It was found in this respect that the DRP-1 mutants carrying serine to aspartic acid substitution displayed 100-fold lower affinity to CaM than WT protein. Conversely, mimicking dephosphorylation by the substitution of Ser<sup>308</sup> to alanine increased the binding affinity to CaM even further.

In parallel, and most surprisingly, it was found that Ser<sup>308</sup> phosphorylation also inhibited DRP-1 homodimerization while the reverse mutation elevated the abundance of dimers and higher oligomeric forms of DRP-1 [9]. The C-terminal tail of DRP-1 is required for its homodimerization as documented by fractionation of DRP-1 mutants on gel filtration columns. The direct CaM binding and in vitro MLC phosphorylation assays proved that the ability to properly dimerize positively affected the binding affinity to CaM. This introduced a second, previously uncharacterized, layer of regulation, in which the dimerization affects the binding to CaM, by imposing, for example, a conformational change in the CaM regulatory segment, which facilitates the interactions with the CaM molecule. Thus, by finding that the status of Ser<sup>308</sup> phosphorylation also affected the dimerization of the protein, it is further suggested that these two events are interconnected and control the CaM responses and enzyme activation. Further analysis of DRP-1 mutants capable of dissecting between phosphorylation and dimerization events, combined with molecular modeling of the catalytic domain, established a novel concept of safety ‘double-locking’ mechanism for silencing the kinase [9].

At the cellular level, DRP-1 autophosphorylation was reduced and dimerization was increased in response to the

external apoptotic signals of Fas or TNF- $\alpha$ , suggesting that these two interconnected events are target for regulation [9].

#### 4. General conclusions and structural speculations

A model structure of the highly homologous catalytic domains of DAPk and DRP-1 was constructed based on the X-ray structure of phosphorylase kinase in complex with a substrate peptide (PDB code 2phk). Of note, the model structure of the catalytic domain perfectly matches the recently published X ray structure of DAPk [15], at the relevant sites which are discussed below. The model predicts that the peptide derived from the CaM-regulatory region of each kinase binds in the active site with Ser<sup>308</sup>-PO<sub>3</sub> positioned next to the ATP binding P-loop (Fig. 4). According to this model, the phosphate moiety of Ser<sup>308</sup> interacts favorably with this loop and with the positively charged Lys<sup>141</sup>. Thus, the ionic interactions between these residues may stabilize the phosphorylated CaM regulatory domain in a locked position in the catalytic cleft. It is therefore speculated that the autophosphorylation at the basal state serves as a locking device ensuring the activation of the kinase only upon the appropriate apoptotic signal and decreasing the chances that the enzyme will be activated by random fluctuations in cellular Ca<sup>2+</sup> levels.

In DRP-1, a second layer of regulation was introduced by this common Ser<sup>308</sup> phosphorylation which involves the homodimerization state of the protein. The phosphorylation on this site prevents the conversion into the dimer conformation that unexpectedly displays its own contribution to the CaM binding. Each of these two steps, dimerization and autophosphorylation, is necessary, yet not sufficient by

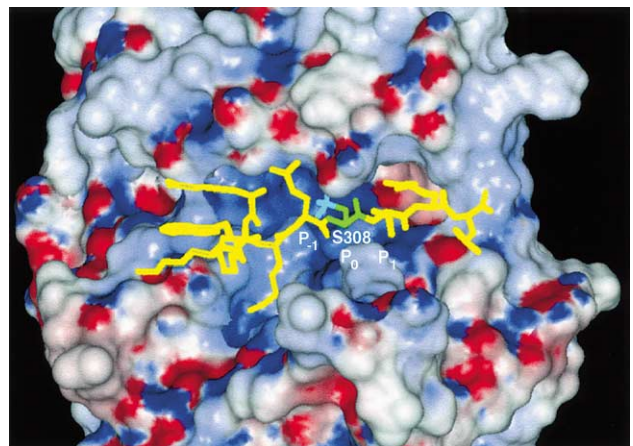


Fig. 4. Three-dimensional model structure of the kinase domain of DRP1/DAPk with a bound peptide derived from the CaM-regulatory segment. The water-accessible surface of the kinase domain is colored according to the electrostatic potential: red for negative and blue for positive potential. The peptide is shown as a stick diagram with Ser<sup>308</sup> emphasized in green and the phosphate moiety in magenta.

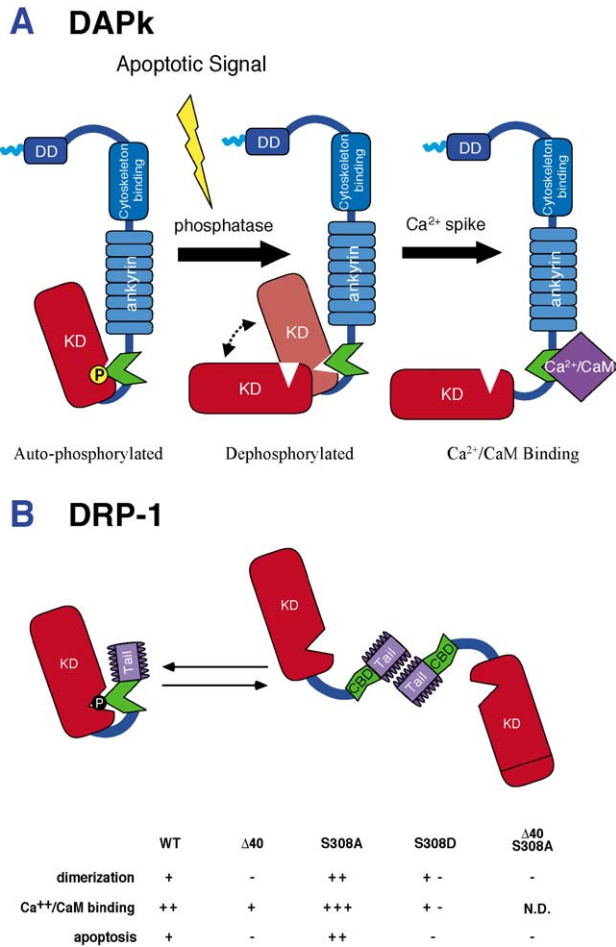


Fig. 5. Specific features that characterize the mode of DAPk and DRP-1 activation. (A) DAPk activation in apoptosis. A proposed mechanism for DAPk regulation consisting of autophosphorylation-based mechanism which imposes a safety device on the catalytic activity of DAPk and consequently restrains its pro-apoptotic functions in growing cells. The functional autophosphorylation site is dephosphorylated upon the apoptotic stimulus leading to DAPk activation. The various domains are marked: KD (kinase domain); DD (death domain). The catalytic cleft is marked by a V-shaped structure in which the phosphate residue on serine 308 resides. (B) DRP-1 activation in apoptosis. The scheme provides a summary of the different features which change by the point mutations or the deletions including the dimerization, CaM binding and apoptotic functions. The various domains are marked by KD (kinase domain); CBD (CaM-binding domain); tail (the C-terminal 40 amino acids peptide). The catalytic cleft is marked by a V-shaped structure in which the phosphate residue on serine 308 resides. N.D., not done.

itself to fully activate the enzyme. We propose that this unique ‘second locking’ mechanism was added on top of the former ‘catalytic block’, which is common to both DAPk and DRP-1. It evolved in DRP-1 as an additional precaution to prevent its inappropriate activation, which may be hazardous to cells. It implies that the process of enzyme activation is very unique and depends on two unlocking steps. One comprises the relief of the CaM-regulatory domain from the catalytic cleft, where it was buried via its negative charge, and the second is the

homodimerization process which may induce additional conformational changes in the CaM-binding domain which facilitate the binding to the activator.

In cells, upon triggering of death signals by the addition of ceramide, Fas or TNF- $\alpha$ , the restraints imposed by the negative autophosphorylation of Ser<sup>308</sup> are removed. This may be achieved by Ser<sup>308</sup> dephosphorylation by a specific phosphatase, the identity of which is yet unknown. In both kinases, the dephosphorylated state results in highly active enzymes with enhanced cell-killing capacity. We propose now that this super-killing activity emerges from increased CaM-independent activity as a result of “weakened lock” (in the case of DAPk) and increased binding activity to CaM, which facilitates full activation even at low Ca<sup>2+</sup>/CaM levels (in the case of both kinases). This enhanced sensitivity to suboptimal levels of Ca<sup>2+</sup>/CaM could potentiate the response of these two kinases to the Ca<sup>2+</sup> spike that takes place during cell death and could also serve to widen the window of kinase activation beyond the time where intracellular Ca<sup>2+</sup> levels are maximal. A scheme of our current understanding of DAPk and DRP-1 activation during cell death is shown in Fig. 5.

Finally, this study opens up a series of very exciting questions to be studied in the future. Once the crystal structures of the catalytic domains of DAPk and of DRP-1, together with their CaM regulatory domains, will be determined (both in the activated and non-active states), this intriguing mechanism of autoinhibition will be deciphered at the atomic level. The specific structural features, which are responsible for this unique autoinhibitory mechanism, may provide an interesting common hallmark for these two kinases which should distinguish them from the other CaM-regulated kinases. Another interesting issue relates to the finding that DAPk and DRP-1 are co-expressed within the same cells and tissues. Thus, the finding that these two different death kinases share a common regulatory process suggests that they may participate in some common signaling pathways. Questions such as whether there exist functional cross interactions between DAPk and DRP-1, or between their specific downstream substrates, should be addressed in the future. Another challenge will be to identify the putative phosphatase that may activate these kinases during cell death.

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