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The regulation of death-associated protein (DAP) kinase in apoptosis



Abstract

DAP-kinase is a calcium/calmodulin ($\text{Ca}^{2+}/\text{CaM}$) serine/threonine kinase which positively mediates programmed cell death in a variety of cell systems. The kinase is localized to the actin microfilament and has a unique, multidomain structure consisting of ankyrin repeats and a death domain. One of the substrates of DAP-kinase was identified as myosin light chain (MLC), the phosphorylation of which mediates membrane blebbing. Another arm in its mode of action leads to the formation of autophagic vesicles. Recent work addressed its mode of regulation and identified a mechanism which restrains its apoptotic function in growing cells and enables its activation during cell death. It

involves an inhibitory type of autophosphorylation on serine 308 within the CaM regulatory domain. This negative phosphorylation takes place in growing cells and is strongly reduced upon their exposure to the apoptotic stimulus of C6-ceramide. The substitution of serine 308 to alanine, which mimics the ceramide-induced dephosphorylation at this site, increases Ca^{2+} /CaM-independent substrate phosphorylation, as well as binding and overall sensitivity of the kinase to CaM. At the cellular level, it strongly enhances the death-promoting activity of the kinase. 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. We propose that this unique mechanism of auto-inhibition evolved to impose a locking device which keeps DAP-kinase silent in healthy cells and ensures its activation only in response to apoptotic signals.

Full text

Introduction

Apoptosis is a genetically controlled, cell death process, which is important at various developmental stages, as well as for 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 [2]. DAP-kinase (DAPk) was discovered using a functional approach to gene cloning, based on transfection of mammalian cells with an anti-sense cDNA library, and subsequent isolation of death protective cDNA fragments [2, 3]. Anti-sense DAPk RNA expression protected HeLa cells from interferon- γ -induced cell death. DAPk is a pro-apoptotic Ser/Thr kinase that participates in a wide array of apoptotic signals including IFN- γ , TNF- α , Fas, activated c-Myc, TGF- β and detachment from extracellular matrix [2, 4-6]. This microfilament-associated Ca^{2+} /calmodulin (Ca^{2+} /CaM)-dependent kinase has a unique multidomain structure that mediates protein-protein interactions such as ankyrin repeats, and the death domain. The death promoting effects of DAPk depend on its catalytic activity, the correct intracellular localization, and on the presence of the death domain [2].

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 undertaken 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, 5, 7-9], the mechanism of DAPk activation has only recently been deciphered in detail (Figure 1).

Catalytic properties of DAPk

The identification of the 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 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 has an auto-inhibitory effect on the catalytic activity and is relieved by binding to Ca²⁺-activated CaM [4]. Consistently, the mere deletion of this segment from DAPk (DCaM 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-inhibition, a second layer of regulation that is mediated by an autophosphorylation mechanism was recently revealed as detailed below [10]. It emerged from previous work, which showed that DAPk undergoes autophosphorylation [4]. Since the pro-apoptotic effect of DAPk depends on its catalytic activity, we wondered whether any of these autophosphorylation sites may reside in the kinase domain, and whether they provide an additional layer of regulation, modulating the catalytic activity. We mapped a functionally relevant autophosphorylation site to Ser³⁰⁸ which resides within the CaM regulatory segment of the enzyme. *In vitro* kinase assays revealed that the autophosphorylation of Ser³⁰⁸ is Ca²⁺/CaM-independent and is strongly inhibited by the addition of Ca²⁺/CaM [10]. The biochemical implication and functional relevance of the phosphorylation status of Ser³⁰⁸ was established in this study by two, single point mutations mimicking the different phosphorylation forms of this specific site. DAPk in which Ser³⁰⁸ was converted

to alanine served to simulate a dephosphorylated state of Ser³⁰⁸, while the substitution to aspartic acid mimicked the phosphorylated form of Ser³⁰⁸ [10].

Biochemical implications of autophosphorylation on Ser³⁰⁸

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 Ca²⁺/CaM-independent catalytic activity upon dephosphorylation (Ser to Ala substitution). This Ca²⁺/CaM-independent activity was minimized by the Ser to Asp substitution [10]. It is suggested that Ser³⁰⁸ 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 Ca²⁺/CaM-independent activity. In fact, a model structure of the catalytic domain of DAPK, which was constructed based on the X-ray structure of phosphorylase kinase in complex with a substrate peptide (PDB code 2phk), predicts that the peptide derived from the CaM-regulatory region binds in the active site with Ser³⁰⁸-PO₃ positioned next to the ATP binding P-loop [10]. According to this model, the phosphate moiety of Ser³⁰⁸ interacts favorably with this loop and with the positively charged Lys¹⁴¹. Thus, the ionic interactions between these residues may stabilize the phosphorylated CaM regulatory domain in a locked position in the catalytic cleft.

The second biochemical property, which is governed by the Ser³⁰⁸ phosphorylation status is the CaM binding activity. The prevention of Ser³⁰⁸ phosphorylation resulted in a marked elevation in CaM binding activity as assessed by two different CaM binding assays *in vitro* [10]. Conversely, the opposing substitution to aspartic acid very 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 which permits a better interaction with CaM. Thus, we concluded that phosphorylation of Ser³⁰⁸ inhibits different aspects of the catalytic activity of DAPK *in vitro*.

DAPK in apoptosis

Next it became of interest to test whether DAPk is subjected to dephosphorylation in response to an apoptotic stimulus in cells. We verified the Ser³⁰⁸ autophosphorylation *in vivo* by using anti-phospho-serine antibodies, and showed that DAPk indeed undergoes autophosphorylation at Ser³⁰⁸ in growing cells. Of importance was the finding that Ser³⁰⁸ undergoes dephosphorylation in response to ceramide, which utilizes active DAPk as part of its mode of action in some cells [10].

The cellular relevance of the two phosphorylation forms of DAPk *in vivo*, i.e. being autophosphorylated in growing cells and dephosphorylated upon apoptotic stimuli, 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 growing 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 'gain of function' super-killer mutant. Thus, phosphorylation on Ser³⁰⁸ silences the pro-apoptotic activity, whereas dephosphorylation of Ser³⁰⁸ is part of the activation process of DAPk [10].

CONCLUSION

We propose a mechanism in which autophosphorylation at the basal state serves as a locking device ensuring the activation of the kinase only upon the appropriate apoptotic signal. The localization of Ser³⁰⁸ within the catalytic cleft promotes its phosphorylation, thereby simultaneously reinforcing the existing "locked" conformation and decreasing the possibility that the enzyme will be activated by random fluctuations of cellular Ca²⁺ levels. Upon triggering of death signals by the addition of ceramide, the restraints imposed by the negative autophosphorylation of Ser³⁰⁸ are removed. This Ser³⁰⁸ dephosphorylation may be achieved by a specific phosphatase, the identity of which is as yet unknown. The dephosphorylated state results in a highly active enzyme with enhanced cell killing capacity. We propose now that this super-killing activity emerges from increased CaM-independent activity of the kinase as a result of "weakened lock" and increased binding activity to CaM which facilitates its full activation even at low Ca²⁺/CaM levels. This enhanced sensitivity to suboptimal levels of Ca²⁺/CaM could potentiate the response of DAPk to the Ca²⁺ spike that takes place during

cell death and could also serve to widen the window of DAPk activation beyond the time where intracellular Ca^{2+} levels are maximal.

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