DAP-kinase is a Ca^{2+} /calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity

Ofer Cohen, Elena Feinstein and Adi Kimchi¹

Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

¹Corresponding author

O.Cohen and E.Feinstein contributed equally to this work

DAP-kinase was initially identified as a gene whose anti-sense-mediated reduced expression protected HeLa cells from interferon- γ -induced programmed cell death. It was cloned in our laboratory by a functional gene selection approach. According to its amino acid sequence, this 160 kDa protein was predicted to be a novel type of calmodulin-regulated serine/threonine kinase which carries ankyrin repeats and the death domain. In this work we have shown that the kinase was autophosphorylated and capable of phosphorylating an exogenous substrate in a Ca^{2+} /calmodulin-dependent manner. We proved that calmodulin binds directly to the recombinant kinase, and generated a constitutively active kinase mutant by the deletion of the calmodulin-regulatory domain. By immunostaining and biochemical fractionations we demonstrated that the kinase is localized to the cytoskeleton, in association with the microfilament system, and mapped a region within the protein which is responsible for binding to the cytoskeleton. Several assays attributed a cell death function to the gene. Ectopic expression of wild-type DAP-kinase induced the death of target cells, and the killing property depended strictly on the status of the intrinsic kinase activity. Conversely, a catalytically inactive mutant that carried a lysine to alanine substitution within the kinase domain, displayed dominant-negative features and protected cells from interferon- γ -induced cell death. DAP-kinase is therefore a novel cytoskeletal-associated cell death serine/threonine kinase whose activation by Ca^{2+} /calmodulin may be linked to the biochemical mechanism underlying the cytoskeletal alterations that occur during cell death.

Keywords: calcium/calmodulin/cytoskeleton/programmed cell death/serine-threonine kinase

Introduction

Programmed cell death (PCD) can be triggered externally through the activation of various cell surface receptors. Among the physiological triggers of PCD, a group of cytokines plays a crucial role, including both diffusible cytokines such as TNF- α , interferon- γ (IFN- γ) and TGF- β , and membrane-bound proteins such as the ligand to Fas/APO-1 receptor (Laster *et al.*, 1988; Trauth *et al.*, 1989; Itoh *et al.*, 1991; Lin and Chou, 1992; Novelli *et al.*,

1994). These findings led to the concept that exposure of cell cultures to a specific cytokine may provide a well-controlled *in vitro* system for the isolation of genes that mediate PCD. Yet, the great potential that resides in this approach began to be exploited only recently. One line of research was directed towards cloning genes that code for proteins that interact with the intracellular domain of death-inducing receptors such as p55 TNF receptor 1 and the Fas/APO-1 receptor. This approach employed the yeast two-hybrid selection system for the rescue of proteins that interact directly with the death domain that constitutes the functional intracellular regions of these receptors. It led to the isolation of a few novel death-domain-containing proteins, FADD/MORT-1, RIP and TRADD, that function as critical mediators of PCD (Boldin *et al.*, 1995; Chinnalyan *et al.*, 1995; Heu *et al.*, 1995; Stanger *et al.*, 1995; Tewari and Dixit, 1996). A second approach, pioneered in our laboratory, employed a functional selection strategy of gene cloning (Deiss and Kimchi, 1991). Using this approach, several novel genes, named DAP genes (for Death Associated Proteins), that function as potential positive mediators of IFN- γ -induced cell death, were isolated and initially characterized (Deiss *et al.*, 1995; Kissil *et al.*, 1995). Other laboratories adapted similar functional selection strategies and candidate genes that mediate cell death induced by IL-3 deprivation or by T-cell receptor activation were cloned including Requiem (Gabig *et al.*, 1994), ALG-2 and ALG-3 (Vito *et al.*, 1996).

Our method, called Technical Knock Out (TKO), was based on random inactivation of genes via introduction of anti-sense cDNA expression libraries, prepared from a mixture of non-treated and IFN- γ -treated cells. The genes of interest were selected and cloned by virtue of the defined phenotypic change—reduced susceptibility to the cytokine-induced cell death—which resulted from their inactivation. HeLa cells, transfected with Epstein-Barr virus (EBV)-based vectors carrying anti-sense cDNA libraries, were subjected to positive selection of cells that survived in the continuous presence of IFN- γ . The rescued plasmids that were positively scored in a second round of transfection carried six non-overlapping groups of cDNA fragments. Sequence analysis indicated that five of them corresponded to novel genes: DAPs 1–5 (Deiss *et al.*, 1995). The sixth gene was identical to a known aspartic protease, i.e. cathepsin D, the participation of which was thereafter established in a variety of apoptotic systems (Deiss *et al.*, 1996).

Our efforts so far have focused on the sequencing and initial characterization of three of the DAP proteins (DAP-1, -2 and -3). The full-length sense cDNAs were isolated and the deduced amino acid structure of the three proteins was determined. Antibodies were raised and the expression of the proteins was studied. It was found that DAP-1 codes for a 15 kDa proline-rich basic protein, that

DAP - Kinase

Fig. 1. Schematic representation of DAP-kinase and its mutants used in these studies. The various motifs and domains as predicted by the deduced amino acid sequence and/or experimental work are shown. The numbers below indicate the amino acid positions. The K42A, Δ CaM and (1–1271) Δ DD mutants are presented schematically below.

DAP-2 is a structurally unique 160 kDa serine/threonine protein kinase (therefore named DAP-kinase), and that DAP-3 codes for a 46 kDa protein that carries a potential ATP/GTP binding motif (Deiss *et al.*, 1995; Kissil *et al.*, 1995).

The deduced amino acid structure of DAP-kinase suggested that a novel prototype of serine/threonine kinase has been isolated. Its kinase domain, composed classically of 11 subdomains, typical of serine/threonine kinases, is followed by a region that shares high homology with the calmodulin regulatory domains of other kinases. Adjacent to the latter, we found eight ankyrin repeats followed by two P-loop motifs. Moreover, a typical death domain module was identified at the 3' end of the protein, followed by a stretch of amino acids that is rich in serines and threonines (Feinstein *et al.*, 1995). The death domain of DAP-kinase has all the boxes of homology and the conserved amino acids characteristic of the analogous domains in the aforementioned death domain-containing proteins. Altogether, the predictions made on the basis of the deduced amino acid sequence suggested that a structurally unique kinase has been identified, and stressed the importance of studying this novel death associated protein both at the biochemical and functional levels.

In this work we describe a series of biochemical assays that confirmed the predictions initially made on the basis of the amino acid sequence analysis. We demonstrated that the kinase is autophosphorylated and is capable of phosphorylating an exogenous substrate in a Ca^{2+} /calmodulin-dependent manner. The direct binding of calmodulin to the recombinant kinase was proven, and a constitutively active mutant kinase was generated by the deletion of the calmodulin-regulatory domain. The biochemical work was complemented by a few functional assays. It was found that ectopic expression of wild-type DAP-kinase induced the death of HeLa cells. The constitutively active mutant kinase had stronger growth-restrictive effects, whereas a catalytically inactive mutant that carries a lysine to alanine substitution within the kinase domain was not cytotoxic in these assays. The latter DAP-kinase mutant displayed dominant-negative features and protected cells from IFN- γ -induced cell death.

Immunostaining and biochemical fractionations revealed that the kinase is localized to the cytoskeleton in association with the microfilament system and defined a region downstream of the first P-loop motif which contributes to this tight association. Interestingly, at the early steps of cell death, changes in actin cytoskeleton organization and in cell morphology preceded the nuclear condensation and segmentation both in response to IFN- γ and upon ectopic expression of DAP-kinase. Thus, the intracellular localization of DAP-kinase may provide a mechanistic clue as to how external death signals impose the cytoplasmic changes that occur during cell death.

Results

Assessment of *in vitro* DAP-kinase activity

The deduced amino acid structure of DAP-kinase protein predicts a few functional motifs and domains as depicted in Figure 1. The N-terminus is composed of a protein kinase domain of the serine/threonine type (Deiss *et al.*, 1995), that spans 255 amino acids from position 13 to position 267. In order to measure the kinase activity we developed an *in vitro* immune complex kinase assay for DAP-kinase. FLAG-tagged wild-type DAP-kinase or DAP-kinase mutants were transiently expressed in COS cells. DAP-kinase proteins were immunoprecipitated by the anti-FLAG antibodies and were subjected to an *in vitro* kinase assay, in the presence of 0.5 mM Ca^{2+} and 1 μM recombinant calmodulin (CaM). Two mutant versions of DAP-kinase were used in this experiment: a C-terminus-truncated DAP-kinase that lacks the last 152 amino acids, a region that contains the death domain and the serine/threonine-rich stretch of amino acids (Feinstein *et al.*, 1995) (named DAP-kinase 1-1271 Δ DD; Figure 1) and a mutant in which a conserved lysine in the kinase subdomain II (at position 42) was substituted with alanine (DAP-kinase-K42A). The latter mutation was shown in other kinases to interfere with the phosphotransfer reaction, giving rise to a catalytically inactive protein (Hanks and Quinn, 1991). As can be seen in Figure 2, the recombinant DAP-kinase protein that was present in the immune complex was phosphorylated *in vitro* resulting in a

Fig. 2. *In vitro* kinase activity of the DAP-kinase. DAP-kinase or DAP-kinase mutant proteins were assayed *in vitro* for kinase activity in the presence of Ca^{2+} /CaM and MLC as described in Materials and methods. The proteins were run on 11% SDS-PAGE and blotted onto nitrocellulose membrane. The upper and the middle panels show the autophosphorylation of DAP-kinase and MLC phosphorylation, respectively, as seen after exposure to X-ray film. The lower panel shows the DAP-kinase proteins by incubation of the same blot with anti-FLAG antibodies and ECL detection.

prominent ^{32}P -labelled band at the expected protein size. In contrast, the mutant DAP-kinase-K42A failed to be phosphorylated, suggesting that the mutation indeed inactivated the enzyme, and that the label of DAP-kinase resulted from autophosphorylation. The homology of the kinase domain of DAP-kinase to the myosin light chain kinase (MLCK; Deiss *et al.*, 1995) prompted us to test the myosin light chain (MLC) as a potential exogenous substrate for the *in vitro* DAP-kinase assays. As can be seen in Figure 2, DAP-kinase, but not its catalytically inactive mutant (DAP-kinase-K42A), phosphorylated the MLC under the *in vitro* kinase assay conditions. The truncated ΔDD mutant, DAP-kinase-1–1271, was capable of undergoing autophosphorylation as well as phosphorylating the MLC. This indicates that: (i) the region of the C-terminus, especially the most terminal stretch of amino acids that is rich in serines and threonines (Feinstein *et al.*, 1995), is either not subjected to autophosphorylation or most probably is not the sole target for that activity; and (ii) the 152 C-terminal amino acids do not participate in recognition of the MLC as a substrate. The amount of the recombinant DAP-kinase protein present in each immune complex was determined by reacting the blots, after the visualization of the ^{32}P signals, with anti-FLAG antibodies (Figure 2).

DAP-kinase is a calmodulin-regulated serine/threonine kinase

A region, located downstream to the kinase domain (amino acids 280–312; Figure 1), was predicted to bind CaM, based on sequence homology with the CaM-regulatory domains of several members of the CaM-dependent kinase family (Deiss *et al.*, 1995). Several different assays performed in the course of this work confirmed both the binding of CaM to the DAP-kinase protein and the regulation of the kinase activity by CaM.

The ability of DAP-kinase to bind CaM was first tested by using labelled CaM in an overlay binding assay. In

this assay, various FLAG-tagged recombinant DAP-kinase constructs were expressed in COS cells and the protein extracts were electrophoresed on SDS-PAGE, blotted onto nitrocellulose membranes, and reacted with [^{35}S]methionine-labelled recombinant CaM (Baum *et al.*, 1993). The wild-type DAP-kinase was tested, as well as a deleted version of DAP-kinase that lacks the calmodulin regulatory and binding region (i.e. amino acids 266–312; named DAP-kinase- ΔCaM), and the previously mentioned ΔDD mutant. The same blots were also reacted with anti-FLAG antibodies to confirm the presence of the recombinant protein appearing at the predicted size in each slot. Both the wild-type DAP-kinase and the truncated ΔDD were capable of binding the labelled CaM, whereas the DAP-kinase- ΔCaM failed to do so (Figure 3A).

The ability of DAP-kinase to bind CaM was further confirmed by using the yeast two-hybrid selection system (Fields and Sternglanz, 1994). In this assay, the region comprising the end of the kinase domain, the CaM regulatory region, the ankyrin repeats domain and the first P-loop (see Figure 1 for details), was used as a bait to fish interacting proteins from the HeLa expression cDNA library (Clontech). About 90 positive clones were obtained, all of them identical to the human CaM full-length cDNA. The rescued CaM clones also reacted in the yeast system with a truncated construct of DAP-kinase which exclusively comprised the end of the kinase domain and the CaM regulatory domain (amino acids 251–364) (E.Feinstein and A.Kimchi, unpublished results). Altogether, the CaM overlay assays, and the interactions between DAP-kinase fragments and calmodulin in the yeast two-hybrid system, confirmed the prediction that DAP-kinase binds CaM through the conserved domain that lies downstream to the kinase domain.

The Ca^{2+} /CaM regulation of the kinase activity was further investigated in *in vitro* kinase assays. In the absence of Ca^{2+} /CaM, both autophosphorylation and MLC phosphorylation by DAP-kinase were 8- to 10-fold lower than phosphorylation in the presence of Ca^{2+} /CaM (Figure 3B). Interestingly, the CaM regulatory domain deletion mutant (DAP-kinase- ΔCaM) displayed a high level of enzymatic activity in the absence of Ca^{2+} /CaM, suggesting a negative regulatory function of this region that could be relieved by the interactions with calmodulin (Figure 3C). These results were consistent with the stimulatory effects imposed by deletion of this region in other CaM-dependent kinases (Shoemaker *et al.*, 1990). We therefore concluded from *in vitro* kinase assays that the kinase activity of DAP-kinase is regulated by Ca^{2+} /CaM, and that removal of the CaM regulatory domain generates a deregulated kinase that is constitutively active.

Ectopic expression of DAP-kinase induces the death of HeLa cells

The first indication that attributed a function to DAP-kinase, as a positive mediator of cell death, was based on the finding that its reduced expression by the anti-sense RNA protected HeLa cells from apoptotic cell death initiated by the IFN- γ receptors. It was therefore interesting to test whether elevated levels of DAP-kinase protein, generated by the ectopic expression of the full-length sense cDNA, may cause cell death directly, without any external stimulus.

Fig. 3. DAP-kinase binds CaM and its activity is regulated by CaM/Ca²⁺. (A) CaM overlay on DAP-kinase. The upper panel displays the results of hybridization with [³⁵S]methionine-labelled recombinant CaM. The lower panel shows the results of hybridization of the same blot with anti-FLAG antibodies to detect the DAP-kinase protein. (B) Ca²⁺/CaM regulation of DAP-kinase activity. DAP-kinase was subjected to *in vitro* kinase assay as described in Materials and methods, in the presence or absence of Ca²⁺ and CaM (the reaction was stopped after 15 min for detecting the autophosphorylation, or after 2 min to measure MLC phosphorylation). The lower panels show the results of incubation with anti-FLAG antibodies (in the same blot). (C) DAP-kinase ΔCaM activity is maximal in the absence of Ca²⁺/CaM. Details are as in (B).

In order to express DAP-kinase in mammalian cells, the full-length cDNA was cloned into pcDNA3 vector (Invitrogen), under the control of the cytomegalovirus (CMV) promoter. Similar constructs were prepared containing the catalytically inactive DAP-kinase-K42A mutant, and the CaM regulatory domain deletion mutant (DAP-kinase-ΔCaM). DAP-kinase constructs, as well as the empty vector, were transfected into HeLa cells by a calcium phosphate co-precipitation technique. After 2–3 weeks of growth in selection medium (G-418), the drug-resistant cells were stained with crystal violet. It was found that transfection with the wild-type DAP-kinase significantly reduced the number of surviving colonies compared with transfections with the empty vector (Figure 4A). The inhibitory effect was even more pronounced upon transfection with the constitutively active DAP-kinase-ΔCaM mutant, suggesting that this mutant had the most prominent growth-restrictive effects. In contrast, the catalytically inactive DAP-kinase mutant did not reduce the number of colonies at all. Rather, the number of colonies generated by transfection with the K42A mutant was slightly increased, compared with transfection with the empty vector, and the size of individual colonies was often larger (Figure 4A). Transfection with the catalytically inactive DAP-kinase mutant therefore seemed to confer some growth advantage to cells during the process of colony formation. These results were repeated in six independent experiments, with different preparations of plasmid DNA. They were also repeated with other types of expression vectors (not shown). These data provided the first indication that ectopic expression of DAP-kinase was not compatible with continuous cell growth, and that this feature depended on intrinsic kinase activity. They also provided the first hint that the catalytically inactive

mutant of DAP-kinase may have a dominant-negative function, an issue examined later under the restrictive effects of IFN-γ (see below).

In order to determine more precisely the fate of the cells and to understand the basis for the suppression of colony formation, the cells were examined 2 days after transfection with the DAP-kinase gene. In these experiments, the LacZ marker gene was used to facilitate recognition of the transfected cells that ectopically express the DAP-kinase. We have constructed for this purpose a vector containing the internal ribosomal entry site (IRES) of poliovirus and thus directing the expression of both LacZ and wild-type DAP-kinase genes within a single bicistronic message. The bicistronic mRNA was expressed from the tetracycline-repressible promoter (Gossen and Bujard, 1992). The morphology of lacZ-containing blue cells was determined 48 h post-transfection, in cultures which were maintained in the absence of tetracycline to allow continuous expression of both genes. It was found that 34% of the lacZ-containing cells which expressed the wild-type DAP-kinase displayed the morphology of apoptotic cells, i.e. cell shrinkage and rounding up followed by detachment from the plates. In contrast, in the control vector a background of <5% apoptotic cells was detected (Figure 4B). Altogether, morphological assessment and colony formation assays suggest that overexpression of DAP-kinase promotes cell death, thus reinforcing the role of DAP-kinase as a positive mediator of cell death.

The catalytically inactive DAP-kinase protects cells from IFN-γ-induced cell death

We next tested the hypothesis that DAP-kinase-K42A mutant may function in a *trans*-dominant-negative manner. This was done by checking whether the catalytically

Fig. 4. Ectopic expression of DAP-kinase induces cell death. (A) HeLa cells (5×10^5 cells/plate) were transfected with 20 μ g DNA of pcDNA3 vector or with DAP-kinase constructs cloned into the same vector. After 48 h, the cell cultures were split 1:5 and subjected to selection with G-418. After 2–3 weeks the plates were stained with crystal violet. (B) HeLa cells (5×10^5 cells/plate) were transfected with 20 μ g DNA of the empty pSBC-bl plasmid, or with the vector which carries the wild-type DAP-kinase. The cells were grown for 48 h in the absence of tetracycline and stained with X-Gal solution. The frequency of blue cells with an apoptotic rounded morphology was assessed by counting 600 total blue cells from six different fields coming from duplicate transfections. The arrow points to a transfected dying cell.

inactive mutant kinase may protect HeLa cells from IFN- γ -induced cell death, similar to the protection conveyed by the anti-sense RNA expression. In this experiment the empty pcDNA3 vector and that containing the DAP-kinase-K42A mutant, were transfected into HeLa cells. At 48 h after transfection, the cells were split and subjected to double selection with 700 μ g/ml G-418 and 200 U/ml IFN- γ . Under these stringent conditions, the transfectants that expressed the control vector were efficiently killed, and the background of G-418-resistant cells was extremely low. In contrast, transfection with the K42A mutant had significantly increased the number of surviving cells (Figure 5A). On average, the relative number of colonies that survived in the presence of IFN- γ was 5-fold higher in the K42A transfectants than in the corresponding pcDNA3 transfectants (Figure 5B). The average cell number per colony was also higher in the K42A transfect-

Fig. 5. DAP-kinase K42A mutant protects HeLa cells from the IFN- γ -induced cell death. (A) HeLa cells (5×10^5 cells/plate) were transfected with 20 μ g DNA of empty pcDNA3 vector or DAP-kinase-K42A cloned into the same vector. After 48 h, the cells were split 1:5 and subjected to selection with G-418 and 200 U/ml of IFN- γ . After 2–3 weeks of selection the plates were stained with crystal violet. Pictures were taken under light microscopy using Kodak TMX100 film (magnification $\times 40$). (B) Number of surviving colonies per 1 cm^2 was counted after the double selection and normalized according to the number of colonies appearing in G-418 selections which were performed in the absence of IFN- γ . Values represent the average of 10 representative fields.

ants. These results indicate that the K42A mutant can protect HeLa cells from IFN- γ -induced cell death, presumably by acting in a dominant-negative manner, interfering with the normal function of the endogenous DAP-kinase.

DAP-kinase is localized to the cytoskeleton

One of the key questions in understanding the mode of action of DAP-kinase concerns its intracellular localization. In order to define, by immunofluorescent staining, the intracellular localization of DAP-kinase, we have transiently transfected SV80 human fibroblasts with the aforementioned FLAG-DAP-kinase-K42A construct, and immunostained the cells with anti-FLAG antibodies. The K42A mutant was chosen, to avoid death-related morphological changes upon overexpression (transfection of SV80 cells with wild-type DAP-kinase induced cell death similar to that observed in HeLa cells, as detailed below). The FLAG-DAP-kinase-K42A was stained as a network of delicate fibres reaching the cell periphery; nuclei were not stained (Figure 6A). The same pattern was also revealed by staining with anti-DAP-kinase monoclonal antibodies (not shown). This was the first indication that suggested a cytoskeletal localization of DAP-kinase protein. Double staining of the transfectants with anti-FLAG antibodies

Fig. 6. DAP-kinase is localized to the cytoskeleton. **(A)** SV80 cells transiently transfected with DAP-kinase-K42A were stained at 48 h with anti-FLAG antibodies and fluorescein-conjugated phalloidin as described in Materials and methods. Both pictures represent the same field (magnification $\times 400$). **(B)** Detergent extraction of HeLa cells. HeLa cells were extracted with 0.5% Triton X-100 to soluble fraction (Sol) and insoluble fraction (InSol) as described in Materials and methods. The protein extracts were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was reacted with anti-DAP-kinase monoclonal antibodies, anti- β -tubulin antibodies and anti-actin antibodies as indicated.

and with fluorescein-conjugated phalloidin which binds to actin fibres, revealed a considerable overlap (Figure 6A). In contrast, there was no overlap with the microtubule staining by anti- β -tubulin antibodies (not shown).

The cytoskeletal localization of DAP-kinase was subsequently confirmed by the biochemical fractionation of both the endogenously and exogenously expressed protein. We used the well-elaborated protocol of gentle cell extraction with non-ionic detergent (0.5% Triton X-100) that removes lipids and soluble proteins, leaving intact the detergent-insoluble matrix composed of the nucleus, the cytoskeleton framework and cytoskeleton-associated proteins. In non-transfected HeLa cells, the endogenous DAP-kinase (recognized by monoclonal antibodies raised against the C-terminus of the protein) appeared exclusively in the detergent-insoluble fraction (Figure 6B). In contrast, β -tubulin and actin, both of which have a constant soluble pool, were each found in the detergent-soluble and -insoluble fractions. We used nocodazole, a microtubule-disrupting drug, to change the solubility of β -tubulin. As can be seen in Figure 6B, after nocodazole treatment of HeLa cells, all of the β -tubulin protein was found in the soluble fraction, whereas the solubility of either DAP-kinase or actin did not change. In contrast, after treatment

of cells with latrunculin A, a microfilament-disrupting agent (Bershadsky *et al.*, 1995), a substantial portion of DAP-kinase was found in the soluble fraction. Here, actin was found almost exclusively in the soluble fraction, whereas the solubility of β -tubulin was not affected. These results, in combination with the double immunostaining, suggest that DAP-kinase might be localized to the microfilament system of the cytoskeleton.

The detergent extraction assay was further used to map the region within the DAP-kinase that associates with the cytoskeleton. For this purpose, we used COS cells transfected with FLAG-DAP-kinase, in which the pattern of staining with anti-FLAG antibodies was similar to that observed in the previously studied SV80 and HeLa cells (Figure 7A). A series of constructed DAP-kinase deletion mutants in the pECE-FLAG or pcDNA3-FLAG expression vectors were transfected into COS cells. The transfected COS cells were subjected to detergent extraction as described above, and the immunoblots were reacted with the anti-FLAG antibodies to monitor in each case the elution profile of each DAP-kinase mutant product. The results are summarized in Figure 7B. From the detailed analysis it was concluded that the region comprising amino acids 641–835 contributes to the detergent insolubility

A **B**

Fig. 7. Mapping the region responsible for cytoskeletal binding. **(A)** Immunostaining of recombinant DAP-kinase. COS cells, transfected with pECE-FLAG-DAP-kinase, were immunostained with anti-FLAG monoclonal antibodies as described in Materials and methods (magnification $\times 400$). **(B)** COS cells were transfected with pECE or pCDNA3 vectors carrying either DAP-kinase or DAP-kinase deletion mutants as indicated. The cells were extracted with 0.5% Triton X-100 as described in Figure 6. Detection was carried out with anti-FLAG antibodies. Sol, detergent-soluble fraction; Insol, detergent-insoluble fraction. Schematic representation of DAP-kinase deletion mutants is shown at the left.

of DAP-kinase and therefore may be a critical region responsible for the association with the cytoskeleton. Its deletion interfered with the cytoskeletal association, and conversely, this region by itself was detergent-insoluble. Fragments containing the ankyrin repeats without the cytoskeletal binding domain were completely detergent-soluble.

We next asked whether the intracellular localization of DAP-kinase may be relevant to the cytoskeletal alterations that occur during the IFN- γ -induced death of HeLa cells. Staining of actin with phalloidin showed that, after treatment with the cytokine, complete disruption of microfilament organization took place, and the stress fibres disappeared in the cells that started to round up (Figure 8A). The loss of stress fibres occurred before the typical nuclear alterations, consisting of chromatin condensation and segmentation (Deiss *et al.*, 1995), had taken place. In order to follow the possible effects of DAP-kinase

overexpression on the cytoskeleton network, we used REF-52 fibroblasts possessing a well-organized actin cytoskeleton. The constitutively active FLAG-DAP-kinase- Δ CaM mutant was transiently transfected into these cells. After 48 h, the cells that were positively stained with the anti-FLAG antibodies were examined for both nuclear and cytoskeletal alterations, in comparison with adjacent non-transfected cells. This was achieved by triple staining with DAPI (for nuclei) and phalloidin (for the microfilament system) (Figure 8B). It was found that the FLAG-positive cells displayed an aberrant, rounded phenotype with a disrupted pattern of microfilament staining, that was reminiscent of the cytoskeletal alterations occurring in the IFN- γ -treated cells. No signs of chromatin condensation or fragmentation could be detected at this time in the DAP-kinase-transfected cells (Figure 8B). In contrast, transfections with the truncated, catalytically active DAP-kinase (DAP-kinase- Δ EcoRV; amino acids 1–305 in

Fig. 8. Changes in actin cytoskeletal organization in the IFN- γ -induced cell death and upon ectopic expression of constitutive DAP-kinase. (A) HeLa cells, grown on glass coverslips, were treated with IFN- γ (1000 U/ml) (b) or were left untreated (a). After 4 days the cells were stained with fluorescein-conjugated phalloidin (magnification $\times 1000$). (B) REF-52 cells were transiently transfected with DAP-kinase mutants as indicated. After 48 h the cells were triple-stained with anti-FLAG antibodies, fluorescein-conjugated phalloidin and DAPI as described in Materials and methods (magnification $\times 100$). The arrows point to the transfected cells.

Figure 7B) which was mislocalized in the cells and showed a nuclear rather than cytoskeletal staining, did not lead to any cytoskeletal alterations. In these transfections, the FLAG-positive cells displayed a normal pattern of microfilament staining which could not be distinguished from adjacent non-transfected cells (Figure 8B). These results were repeated in SV80 cells, in which $>80\%$ of transfectants expressing the FLAG-DAP-kinase- Δ CaM mutant showed abnormal pattern of microfilament staining, whereas transfections with DAP-kinase- Δ EcoRV mutant had no effect (data not shown). These results suggest a link between the correctly localized active DAP-kinase and the cell death-related cytoskeletal and morphological changes that develop in response to IFN- γ .

Discussion

DAP-kinase is a novel prototype of Ca^{2+} /calmodulin-dependent serine/threonine kinase, with a complex structure and a cytoskeletal intracellular localization that may be relevant to its function. In this work, the basic biochemical properties of this 160 kDa kinase were characterized, and its functional relevance to programmed cell death was established. Taken together, the information on the complex structure of the protein and on its cellular function, places the DAP-kinase at an important junction point within the branched network that leads to cell death, where it may receive and release a variety of input and output signals.

In this work we have proved by *in vitro* kinase assays that DAP-kinase has an intrinsic kinase activity towards itself and towards an exogenous substrate (MLC). This activity was blocked by a classical mutation that substituted a critical lysine to alanine within the second kinase subdomain. Future determination of the DAP-kinase phosphorylation sites by phosphopeptide mapping, and

testing their effect on DAP-kinase activity by mutation analysis, should clarify their functional significance. We have also proved in this work that DAP-kinase can bind calmodulin and be activated by this binding. Thus, DAP-kinase appears as an attractive candidate that may couple the death-related changes of intracellular concentrations of Ca^{2+} to a biochemical pathway that imposes cell death. Previous reports have shown that calmodulin inhibitors, and inhibitors of calmodulin-dependent kinases, elicited death-protective effects in different apoptotic systems (Dowd *et al.*, 1991; Wright *et al.*, 1993), suggesting that DAP-kinase is a possible target for these early observations.

DAP-kinase was initially identified as a gene whose anti-sense-mediated reduced expression protected HeLa cells from IFN- γ -induced cell death (Deiss *et al.*, 1995). This was the first indication that linked the function of this gene to programmed cell death. Several additional independent lines of evidence obtained in this work strengthened this notion, suggesting altogether that this novel serine/threonine kinase is a bona fide 'death gene'. First, we found that overexpression of DAP-kinase killed HeLa cells in the absence of any external stimulus. It is well established now, that ectopic expression of a single gene which is part of a pathway that leads to cell death, is often sufficient by itself to trigger this biological process. This was shown with respect to p53 (Haupt *et al.*, 1995), and the different members of the ICE gene family (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994; Tewari *et al.*, 1995), as well as in the case of death-domain-containing proteins that function proximal to the Fas and the p55 TNF receptors, i.e. FADD/MORT1 or TRADD (Boldin *et al.*, 1995; Chinnalyan *et al.*, 1995; Heu *et al.*, 1995; Stanger *et al.*, 1995). In most of the aforementioned cases, elevation of gene expression was not the physiological way in which the protein product was activated

during cell death. Nevertheless, the ectopic expression was effective and provided a strong support for the functional relevance of these genes in death processes. The enhancement of DAP-kinase-induced cell killing by the deletion of the CaM regulatory region (shown in Figure 3C to give rise to a constitutively active kinase) and the complete abrogation of killing by the catalytically inactive kinase, provided direct evidence that both the kinase activity and the Ca^{2+} /CaM regulation of DAP-kinase are crucial for the killing activity of this protein. A second line of evidence connecting DAP-kinase to cell death emerged from the finding that the catalytically inactive K42A DAP-kinase mutant functioned in a dominant-negative manner and protected the HeLa cells from IFN- γ -induced cell death. DAP-kinase carries ankyrin repeats and the death domain, each of which represents a different protein module capable of forming stable complexes with other proteins—probably the downstream or upstream effectors of the kinase (Feinstein *et al.*, 1995). Since we found that the intrinsic kinase activity was critical for the death-promoting properties of the protein, it is possible that the catalytically inactive K42A DAP-kinase mutant may interfere with the function of the endogenous wild-type kinase, by titrating out its effectors through the above-mentioned other functional motifs that K42A still carries. However, we still do not rule out the possibility of kinase homodimerization.

One of the major challenges is the identification of the critical DAP-kinase substrates that become phosphorylated during cell death, and the understanding of how these phosphorylations eventually lead to collapse of the cell. Determination of the intracellular localization is one way that can direct this aspect of the studies. The tight association with cytoskeletal elements responsible for the detergent insolubility of DAP-kinase may suggest, though not exclusively, that cytoskeletal proteins are among the potential substrates. Interestingly, alterations of the actin fibre organization were among the first changes that could be identified in HeLa cells after exposure to IFN- γ (Figure 8A). These alterations clearly preceded the typical nuclear condensation and fragmentation steps previously reported in this system (Deiss *et al.*, 1995). It is possible that the cytoskeletal alterations which obviously contribute to the collapse of cells in PCD, may be directly or indirectly mediated by DAP-kinase, as its overexpression also led to similar cytoskeletal alteration. Obviously, a more detailed analysis of the morphological changes that occur at different time intervals upon promoter activation in inducible stable clones is needed. Use of such clones may elucidate more precisely the sequential steps, imposed by DAP-kinase activation, that lead to the collapse of a cell during apoptosis and may indicate whether the chromatin condensation and segmentation steps are controlled by another, DAP-kinase-independent, pathway. A more detailed immunostaining and biochemical analysis is also required in order to identify the precise components within the microfilament system to which DAP-kinase binds. It should be mentioned in this respect that computer analysis of the ~200 amino acid region of DAP-kinase that was found responsible for the detergent insolubility (Figure 7B) did not reveal any known domains or motifs, or any homology to regions of known proteins that mediate their interaction with the cytoskeleton.

To study the functional position of DAP-kinase with respect to other known mediators of cell death, co-expression assays may be used. For instance, neutralization of the death-inducing effects of DAP-kinase by crmA or p35 (Beidler *et al.*, 1995; Tewari and Dixit, 1995) may indicate that members of the ICE family of proteases function downstream to the DAP-kinase gene. Also, the crosstalk with other DAP genes that we have recently cloned can be assayed in a similar fashion. For instance, testing whether anti-sense DAP-3 RNA (Kissil *et al.*, 1995), or cathepsin D inhibition of expression or function (Deiss *et al.*, 1996) may suppress the DAP-kinase-induced cell death should determine the functional relationships between those genes. Finally, in light of our finding that DAP-kinase is widely expressed in various tissues (E.Feinstein and A.Kimchi, unpublished results), it will be of interest to screen a large spectrum of different cell systems and apoptotic signals for testing the direct involvement of DAP-kinase.

Materials and methods

Cell culture

HeLa human epithelial carcinoma cells, COS-7 monkey kidney cells, SV-80 cells (human fibroblasts transformed with SV40 large T-antigen), and REF-52 rat embryo fibroblasts, were grown in DMEM (BioLab) supplemented with 10% FCS (Gibco), 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. HeLa-tA were grown in the presence of 200 $\mu\text{g}/\text{ml}$ G-418 (Gossen and Bujard, 1992). Transfections were performed by the standard calcium phosphate technique. Recombinant human interferon- γ (3×10^7 U/ml) was purchased from PeproTech. Nocodazole was purchased from Sigma; latrunculin A was a gift from A.D.Bershadsky.

Plasmid construction

DAP-kinase expression constructs for transient transfections into SV80, REF-52 or COS cells and for stable transfections into the HeLa cells were prepared in either the pECE (Deng and Karin, 1993) or pCDNA3 (InVitrogen) vectors. In all constructs the DAP-kinase sequences were tagged with the FLAG epitope at their N-terminus. In C-terminal deletion constructs the DAP-kinase sequences were fused to the FLAG epitope via the *NdeI* restriction site that was introduced at the initiation ATG codon by oligonucleotide-directed mutagenesis. In other constructs, DAP-kinase sequences were fused to the FLAG epitope via the corresponding restriction sites. C-terminal deletion constructs: 1–1271, 1–835, 1–641 and 1–305 were prepared by digestion of the DAP-kinase cDNA with *HindIII* (nt 4146), *XbaI* (nt 2838), *BglIII* (nt 2256) and *EcoRV* (nt 1247), respectively. The full-length DAP-kinase cDNA construct reaches the *EcoRI* site at position 4932 of the 3' UTR. DAP-kinase expression constructs 305–641, 641–835 and 641–1423 contain cDNA fragments obtained by double digestion with *EcoRV* and *BglIII* (nt 1247–2256), *BglIII* and *XbaI* (nt 2256–2838), or by digestion with *BglIII* (nt 2256–4827), respectively. Three DAP-kinase mutants, K42A, ΔCaM and ΔCyt , were prepared using oligonucleotide-directed mutagenesis with the 5'-GTATCCCGCCGCATTCATCAAGA-3', 5'-CAGCATCCCTGG-ATCAAGTCCAGAAGTAACATGAGT-3' and 5'-AAGACGGCAGAA-GATCTAGAAGAGCCCTAT-3' oligonucleotides, respectively. All the nucleotide numbers are given according to X76104.

DAP-kinase was expressed transiently in HeLa cells from the tetracycline-repressible promoter as a bicistronic message with the LacZ sequences. DAP-kinase sequence was tagged with the HA epitope at the N-terminus via the *NdeI* site introduced at the initiation ATG codon. The vector for expression of DAP-kinase–LacZ bicistronic message was prepared by insertion of BH–LacZ fusion gene from pUT535 vector (Cayla) into the *NotI* site of pSBC vector (Kirchhoff *et al.*, 1995). The resulting vector was named pSBC-bl.

Calmodulin overlay

Transfection into COS cells, preparation of cell lysates, SDS–PAGE and transfer of proteins to nitrocellulose, were performed as previously described (Deiss *et al.*, 1995). Protein extracts (300 μg per lane) from

COS cells, non-transfected or transfected with FLAG-DAP-kinase or DAP-kinase mutants were run on 7.5% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was preincubated for 30 min in calmodulin binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl_2) containing 1% non-fat dry milk powder. Recombinant ^{35}S -labelled calmodulin (Baum *et al.*, 1993) was supplemented, and the membrane was subjected to gentle shaking at room temperature for 16 h, washed three times (5 min each) in calmodulin binding buffer, dried and exposed to X-ray film. Detection of FLAG-DAP-kinase was done using anti-FLAG antibodies (1:500; IBI, Kodak) and the ECL Western blotting detection system as described (Deiss *et al.*, 1995).

In vitro kinase assay

Cell lysates of COS transfected cells were prepared as described previously (Deiss *et al.*, 1995). Immunoprecipitation of recombinant DAP-kinase protein from 150 μg total extract was done with 20 μl anti-FLAG M2 gel (IBI, Kodak) in 200 μl of PLB supplemented with protease and phosphatase inhibitors for 2 h at 4°C. Following three washes with PLB, the immunoprecipitates were washed once with reaction buffer (50 mM HEPES pH 7.5, 8 mM MgCl_2 , 2 mM MnCl_2 and 0.1 mg/ml BSA). The proteins bound to the beads were incubated for 15 min at 25°C in 50 μl of reaction buffer containing 15 μCi [$\gamma\text{-}^{32}\text{P}$]ATP (3 pmol), 50 mM ATP, 5 μg MLC (Sigma) and (where indicated) also 1 μM bovine calmodulin (Sigma), 0.5 mM CaCl_2 or 3 mM EGTA in the absence of CaCl_2 . Protein sample buffer was added to terminate the reaction and, after boiling, the proteins were analysed on 11% SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and ^{32}P -labelled proteins were visualized by autoradiography.

Detergent extraction assay

Sub-confluent cultures of COS transfected or HeLa cells, grown on 9 cm plates, were washed once with PBS and then with MES buffer (50 mM MES pH 6.8, 2.5 mM EGTA, 2.5 mM MgCl_2). Where indicated, HeLa cells were pre-treated with 1 $\mu\text{g}/\text{ml}$ nocodazole for 0.5 h, or with 5 μM latrunculin A for 1 h, before extraction. The cells were extracted for 3 min with 0.5 ml of 0.5% Triton X-100 in MES buffer supplemented with protease inhibitors. The supernatant (the soluble fraction, Sol.) was collected, centrifuged for 2 min at 16 000 g at 4°C, and the clear supernatant was then transferred to new tubes. Two volumes of cold ethanol were added and the tubes were incubated at -20°C overnight, centrifuged 10 min at 16 000 g at 4°C and resuspended in 200 μl of 2 $\times$ protein sample buffer without dye. The detergent-insoluble matrix (InSol) remaining on the plate was extracted in 200 μl of 2 $\times$ protein sample buffer, scraped from the plate with rubber policeman and collected into tubes. The samples were loaded on 10% SDS-PAGE; 100 μg protein extracts were loaded on each lane from the Sol fraction, equivalent volumes of InSol were loaded. Analysis of proteins was performed using anti-FLAG antibodies (Kodak), monoclonal antibodies against DAP-kinase (1:1000 dilution; Sigma), anti-tubulin antibodies (1:2000 dilution; Sigma) or polyclonal anti-actin antibodies (1:100 dilution; Sigma) as described above.

Immunostaining of cells

Transfected cells (SV80, REF-52 or COS cells) were plated on glass cover-slips (13 mm diameter), 20 000 cells/well in 1 ml medium within a 24-well plate. After 48 h, the cells were washed twice with PBS, fixed and permeabilized simultaneously. This was carried out by incubating the coverslips for 5 min in a mixture of 3% paraformaldehyde and 0.3% Triton X-100 in PBS, and then incubating with 3% paraformaldehyde alone for an additional 20 min. The cells were washed three times in PBS and then incubated in blocking solution (5% normal goat serum and 1% BSA in PBS) for 60 min. The cells were incubated with 30 μl of the first antibody (anti FLAG 1:300) for 60 min at room temperature, then washed three times in PBS and incubated for 30 min with 30 μl of rhodamine-conjugated goat anti-mouse antibodies (dilution 1:200; Jackson Immuno Research Labs) DAPI (0.5 $\mu\text{g}/\text{ml}$; Sigma) and fluorescein-conjugated phalloidin (1:100; Molecular Probes Inc.) were added at this step. The coverslips were washed three times in PBS, drained and mounted in Mowiol. Microscopy was done under conditions of fluorescent light. Photography was with Kodak TMX400 film.

X-Gal staining

To detect LacZ expression, cells were fixed with 3% paraformaldehyde for 5 min, rinsed twice with PBS and stained for 3 h in X-Gal buffer containing 77 mM Na_2HPO_4 , 23 mM NaH_2PO_4 , 1.3 mM MgCl_2 , 1 mg/ml X-Gal, 3 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 3 mM $\text{K}_4\text{Fe}(\text{CN})_6$. The reaction was

stopped by addition of 70% ethanol. Photography was done under phase microscopy using Kodak Ektachrome 160T.

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