Death-associated Protein (DAP) Kinase Plays a Central Role in Ceramide-induced Apoptosis in Cultured Hippocampal Neurons*

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Treatment of cultured hippocampal neurons with high concentrations of short-chain acyl ceramide derivatives, such as N-hexanoyl-D-sphingosine (C₆-Cer), results in apoptotic cell death. We now show that death-associated protein (DAP) kinase plays an important role in mediating this effect. Upon incubation with C₆-Cer, DAP kinase levels are elevated as early as 1 h after treatment, reaching levels 2-3-fold higher than untreated cells after 4 h. Neurons cultured from DAP kinase-deficient mice were significantly less sensitive to apoptosis induced by C₆-Cer or by ceramide generated via high concentrations of nerve growth factor. A peptide corresponding to the 17 amino acids at the C terminus of DAP kinase protected wild type neurons from C₆-Cer-induced death and from death induced by the addition of exogenous bacterial neutral sphingomyelinase, whereas a scrambled peptide had no protective effect, implying that the DAP kinase C-terminal tail inhibited the function of DAP kinase. Together, these data demonstrate that DAP kinase plays a central role in ceramide-induced cell death in neurons, but the pathway in which DAP kinase is involved is not the only one via which ceramide can induce apoptosis.

Death-associated protein (DAP) kinase is a Ca²⁺/calmodulin-regulated serine/threonine kinase that acts as a positive mediator of apoptosis induced by interferon-γ, Fas, tumor necrosis factor α, detachment from the extracellular matrix, or by deregulated oncogenes such as c-Myc or E2F-1 (1–4). DAP kinase has a multidomain structure that is unique among members of the extended family of calmodulin-dependent protein kinases (5). In addition to the kinase domain and the typical calmodulin regulatory segment, the protein has eight ankyrin repeats, a cytoskeleton binding region, and a conserved death domain (1, 6). The pro-apoptotic function of DAP kinase requires the joint action of several domains (reviewed in Ref. 7) and depends on autophosphorylation (8). One important region was recently discovered by a genetic screen based on functional selection of random short DAP kinase-derived cDNA fragments that protect cells from apoptosis by inhibiting the function of the endogenous full-length protein (9). One of the biologically active DAP kinase fragments corresponded to the last 17 amino acids at the C-terminal of the protein, which immediately follows the death domain. When ectopically expressed, the DAP kinase C-terminal tail peptide inhibited the function of DAP kinase. Its removal from the protein results in enhanced killing activity, indicating that the tail is autoinhibitory, possibly preventing the function of adjacent domains in DAP kinase (9).

In the developing and adult rat central nervous system, DAP kinase mRNA is widely expressed in proliferative and postmitotic regions within the cerebral cortex, hippocampus, and cerebellar Purkinje cells, from embryonic day 13 and onward (10). Expression in the brain is markedly decreased postnatally but remains high in a number of areas, particularly the hippocampus. This temporal and spatial regulation of DAP kinase expression suggests that it may be involved in various neuronal functions, including neuronal cell death. This possibility is supported by the elevation of DAP kinase mRNA levels prior to cell death induced by transient forebrain ischemia (10).

A death-promoting molecule that has received wide attention recently is ceramide, which is produced in response to a number of stimuli in various cell types (11–13). In cultured hippocampal neurons, high concentrations of ceramide, ceramide produced by the activity of neutral sphingomyelinase, or ceramide produced by the binding of nerve growth factor (NGF) to the p75 neurotrophin receptor, induces apoptotic cell death at different stages of neuronal development (14–16). We now show, by comparing the death response of wild type hippocampal neurons to the corresponding DAP kinase null cells, that DAP kinase is involved in mediating the apoptotic effects of ceramide on hippocampal neurons in culture. In addition, a peptide, with a composition identical to the last 17 C-terminal amino acids of DAP kinase blocks the apoptotic response of hippocampal neurons to ceramide and to bacterial sphingomyelinase (bSMase), suggesting that DAP kinase plays an important role in ceramide-induced neuronal cell death.

EXPERIMENTAL PROCEDURES

Hippocampal Cell Cultures—Rat hippocampal neurons were cultured at low density as described (14, 17, 18). Briefly, the dissected hippocampi of embryonic day 18 rats (Wistar) were dissociated by trypsinization (0.25% w/v for 15 min at 37 °C). The tissue was washed in Mg²⁺/Ca²⁺-free Hanks’ balanced salt solution (Invitrogen), and dissociated by repeated passage through a constricted Pasteur pipette. Cells were plated in minimal essential medium with 10% horse serum at a density of 6,000–25,000 cells per 13-mm glass coverslip that had

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† The abbreviations used are: DAP kinase, death-associated protein kinase; C₆-Cer, N-hexanoyl-D-sphingosine; bSMase, bacterial sphingomyelinase; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor.

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been precoated with poly-l-lysine (1 mg/ml). After 2–4 h, coverslips were transferred into 24-well multidishes (Nunc) that contained a monolayer of astroglia as described previously (14, 17, 18). Coverslips were placed with the neurons facing downward and were separated from the glia by paraffin "feet"; these cultures were maintained in serum-free medium (minimal essential medium) which included N2 supplements (17), ovalbumin (0.1%, w/v), and pyruvate (0.1 mM). Hippocampal neurons from embryonic day 17 DAP kinase−/− and wild type (+/+) mice were cultured at low density exactly as described for rat neurons. In some experiments, coverslips were placed in multidishes containing B27-supplemented neurobasal medium (19), and cultures were maintained in this medium in the absence of a co-culture of a glial monolayer. Neurons co-cultured with a glial monolayer or cultured in serum-free medium were harvested and lysed at the indicated time points, and levels of DAP kinase were determined by Western blotting. β-Tubulin levels indicate that similar amounts of protein were loaded in each lane. Two independent experiments are shown.

RESULTS

DAP Kinase Is Up-regulated by C6-Cer—We previously demonstrated that high concentrations of C6-Cer induce cell death in cultured hippocampal neurons via an apoptotic mechanism (14), inasmuch as nuclear condensation and annexin binding were observed, followed by retraction of axons and dendrites and detachment of neurons from the coverslips. In the current study, similar pro-apoptotic effects of C6-Cer were observed on both rat and mouse neurons (not shown).

To determine whether DAP kinase is involved in mediating these effects, neurons were incubated with C6-Cer for various times. DAP kinase expression increased significantly 1 h after treatment and was elevated 2–3-fold after 4 h, remaining at similar levels for up to 8 h (Fig. 1), times which significantly preceded the appearance of apoptotic neurons. This suggests that DAP kinase activation may play a causal role in the onset of apoptosis in neurons.

DAP Kinase Plays an Important Role in C6-Cer-Induced Neuronal Death—To determine the role that DAP kinase plays in ceramide-induced neuronal death, primary hippocampal neurons were cultured from DAP kinase null mice. These mice are homozygous for a targeted inactivating mutation in the DAP kinase gene and hence fail to express DAP kinase (Fig. 2A). When co-cultured with a glia monolayer (see "Experimental Procedures"; here the glia monolayer was physically separated from the neurons) and analyzed after 16 h of treatment with 15 μM C6-Cer, DAP kinase−/− neurons were resistant to C6-Cer-induced apoptotic death compared with wild type neurons, in which ~70% apoptotic neurons were observed at this time point (Fig. 2B).

DAP kinase−/− neurons cultured in B27-supplemented neurobasal medium in the absence of a glial monolayer were also significantly more resistant to C6-Cer-induced apoptotic death. However, at the highest concentrations of C6-Cer tested (15 μM) did not induce death even after 48 h (Fig. 3A), a small but significant increase in cell death was also observed in DAP kinase−/− neurons after 16–22 h, although far less pronounced than in wild type neurons (Fig. 3A). At longer time points (i.e. after 48 h of treatment with C6-Cer), no difference could be detected between wild type and DAP kinase−/− neurons (Fig. 3B), and both were susceptible to C6-Cer-induced cell death. The biologically inactive C6-dihydroceramide (15 μM) did not induce death even after 48 h (Fig. 3B).

Ceramide-induced Apoptosis via Activation of the p75 Neurotrophin Receptor Involves DAP Kinase—We have recently shown that application of high concentrations of NGF to hippocampal neurons that express sufficiently high levels of the p75 neurotrophin receptor results in endogenous ceramide generation and neuronal cell death (2). Application of 500 ng/ml NGF to wild type mouse neurons causes apoptotic cell death as detected by chromatin condensation (Fig. 4, A–D) whereas no chromatin condensation could be detected in neurons from DAP kinase−/− mice (Fig. 4, E–H). Quantification of the extent of chromatin condensation is shown in Fig. 4, I.
A C-terminal Peptide of DAP Kinase Protects Neurons from Apoptosis—We have previously shown that ectopic expression of a cDNA fragment encoding the 17-amino acid tail derived from the C terminus of DAP kinase is the shortest fragment that inhibits the pro-apoptotic functions of DAP kinase (9). To determine the possible role of this C-terminal fragment in ceramide-induced neuronal death, a fluorescent derivative of the peptide was synthesized. This peptide, SCNSGTSYNSISSVVSR, consisting of mainly polar, uncharged amino acids and lacking charged residues, was covalently attached to tetramethylrhodamine isothiocyanate to increase its hydrophobicity. The rhodamine-peptide was internalized into neurons, albeit by an unknown mechanism, and could be detected at high levels in the cytosol as early as 1 h after addition, remaining at high levels for at least 16 h (Fig. 6, A and B). Neurons treated with the peptide were resistant to the apoptotic effects of either C6-Cer or of exogenously added neutral bacterial bSMase (Fig. 7). In contrast, a peptide with the same amino acid composition, but whose sequence was scrambled, did not block cera-
that DAP kinase, a general mediator of cell death that is expressed at high levels in the developing and mature nervous system, plays an important role in mediating neuronal cell death induced by ceramide. This was demonstrated by two complementary approaches, namely analysis of death in neurons cultured from a DAP kinase-deficient mouse and by the use of a C-terminal peptide that inhibits the pro-apoptotic functions of DAP kinase. A central role for DAP kinase is also suggested by the observation that DAP kinase levels are elevated in response to ceramide.

The biological relevance of our findings is underlined by the involvement of DAP kinase in NGF-induced neuronal death via the p75 neurotrophin receptor. Although there is some dispute about the precise role of ceramide in triggering apoptosis, large amounts of evidence have accumulated over the past decade supporting the idea that ceramide, generated either by the activation of sphingomyelinase(s) or by de novo synthesis, is a bona fide second messenger, the levels of which can be modulated by a variety of external signals (11–13). In our study, ceramide induces DAP kinase expression by an as yet unknown mechanism, although we do not know whether the increased expression is the main reason for DAP kinase activation in dying neurons. Likewise, the downstream effectors of DAP kinase, including its direct substrates, are not known, and this issue is being actively pursued in our laboratories.

The lack of complete resistance to ceramide-induced cell death in DAP kinase−/− neurons suggests that although DAP kinase is a central player in ceramide-induced cell death, the pathway in which DAP kinase is involved is not the only one by which ceramide can induce apoptosis. Thus, there could be several pathways of signaling originating from ceramide generation that are activated either by different upstream effectors or at different times or at different intracellular locations. However, we can exclude the possibility that membrane integrity is disrupted by high concentrations of CerH2O-induced long times as CerH2O-dihydroceramide had no effect at similar concentrations and after several times of incubation.

Finally, an important aspect of this study relates to the development of a specific peptide inhibitor, which is internalized by neurons, accumulates within cells, and subsequently efficiently blocks apoptosis. The mechanism of internalization of this peptide is not known, but its cytosolic distribution suggests that it is not internalized by a facilitated mechanism, such as fluid-phase endocytosis, but rather by nonspecific transport across the cell membrane. This peptide inhibitor is being further used to study the function of DAP kinase in apoptosis in other systems and may serve as a starting point to further develop pharmaceutical anti-apoptotic drugs consisting of short, functionally active peptide derivatives and/or of peptide mimetics to modulate apoptotic processes in vivo.

REFERENCES