Calcineurin Potentiates the Activation of Procaspase-3 by Accelerating Its Proteolytic Maturation*

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We have previously shown that procaspase-3 exists in a high molecular weight complex in neonatal rat brain. Here, we purify and identify the protein that interacts with procaspase-3 from rat neonatal cortex. We searched binding proteins to procaspase-3 from a cytosolic extract of neonatal rat brain using chromatogram, two-dimensional gel electrophoresis, and far Western immunoblot. Analysis by tandem mass spectrometry identified the protein as a regulatory subunit of calcineurin (calcineurin B). Overexpression of calcineurin B in HEK293 cells potentiated processing of caspase-3 and apoptosis triggered by tumor necrosis factor-α and cycloheximide treatment. In a cell-free system, overexpression of calcineurin B in HEK293 cells markedly increased processing of caspase-3 by cytochrome c. Immunodepletion of calcineurin B from cytosolic extracts from Jurkat cells decreased processing of caspase-3 by cytochrome c. Knockdown of calcineurin B by RNA interference resulted in reduced apoptosis in HEK293 cells but not in caspase-3-deficient MCF-7 cells. These results suggest that calcineurin B potentiates the activation of procaspase-3 by accelerating its proteolytic maturation.

Apoptosis is a type of cell death resulting from the activation of a genetically regulated cell suicide program. In the nematode Caenorhabditis elegans, a genetic pathway of apoptosis has been identified. Two genes, ced-3 and ced-4, are essential for the execution of apoptosis (1), and ced-9 negatively regulates apoptosis by preventing activation of ced-3 and ced-4 (2). Caspases, human homologues of the C. elegans CED-3, are the key effectors in the execution of apoptosis (3, 4). Caspases are synthesized in cells as inactivezymogens (procaspases), which become proteolytically processed to generate active caspase. Although the exact mechanism that activates caspases is not fully understood, at least two major pathways have been identified: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway involves release of cytochrome c from mitochondria into the cytosol. In the presence of cytochrome c, apoptotic protease activation factor-1 (Apaf-1),2 a human homologue of the C. elegans CED-4, interacts with caspase-9 and produces a multiprotein complex, termed apoptosome (6–10). The assembly of the apoptosome complex represents the initiating step for the activation of the intrinsic caspase cascade. Once activated in the apoptosome, caspase-9 cleaves procaspase-3.

Multiple lines of evidence indicate that Apaf-1, cytochrome c, and caspase-9 are crucial components of the intrinsic pathway. Two other classes of regulators have been reported. The best known of these is the Bcl-2 family, a human homologue of the C. elegans CED-9. The Bcl-2 family consists of both antiapoptotic and proapoptotic members. Antiapoptotic proteins, such as Bcl-2, prevent the release of cytochrome c and thereby inhibit the subsequent activation of caspase (11). However, there is some evidence that Bcl-2 may prevent apoptosis downstream of cytochrome c. In C. elegans, CED-9 directly binds to CED-4 and inhibits the activation of CED-3 (12). It has remained unclear whether Bcl-2 inhibits the function of Apaf-1 by direct binding (13). Another family is the inhibitor of apoptosis (IAP) proteins, such as X-linked IAP. IAPs directly bind to active caspases and either suppress their protease activity or target them for destruction by ubiquitination and subsequent proteasome-mediated degradation (14, 15). X-linked IAP blocks the mitochondrial apoptosis pathway by inhibiting caspase activity directly (16). The activity of X-linked IAP is regulated by release of other factors, such as Smac/Diablo, from mitochondria (17, 18).

To date, no activators have been identified that function subsequent to the release of cytochrome c from mitochondria. Several lines of evidence indicate that heat shock proteins (Hsps) may function as apoptosis activators, but the mechanism remains unclear. Although Hsp70 or Hsp72 suppresses apoptosis by binding to Apaf-1 directly (19–21), Hsp60 has been reported to accelerate the activation of caspase-3, indicating that Hsp60 is proapoptotic (22, 23).

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2 The abbreviations used are: Apaf-1, apoptotic protease activation factor-1; AMC, aminomethylcoumarin; TNF-α, tumor necrosis factor-α; CHX, cycloheximide; Hsp, heat shock protein; CHAPS, 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonic acid; IAP, inhibitor of apoptosis; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid; DSS, dioxidinimidyl suberate; siRNA, small interference RNA.
We have previously shown that procaspase-3 exists in a high molecular weight complex in neonatal rat brain (24). Here, we purified the protein that interacts with procaspase-3 from rat neonatal cortex and identified that it is the regulatory subunit of calcineurin (calcineurin B). In addition, we report the function of calcineurin B in the processing of caspase-3.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human tumor necrosis factor-α (TNF-α) was purchased from R&D systems (Minneapolis, MN). Acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVDD-AMC) was from Peptide Institute (Osaka, Japan). Recombinant procaspase-3 and granzyme B and tacrolimus were from Calbiochem. Recombinant active caspase-9 and anti-caspase-9 antibody were from MBL (Nagoya, Japan). Anti-calcineurin A antibody was from Transduction Laboratories (Lexington, KY). Anti-cleaved caspase-3 antibody was from Cell Signaling Technology (Beverly, MA). Anti-calcineurin B antibody was from Oncogene (Cambridge, MA). Anti-calcineurin was from Upstate (Charlottesville, VA). Anti-calmodulin was from Upstate (Charlottesville, VA). Anti-calmodulin antibody was from Transduction Laboratories, a gift from Dr. Shibasaki, cloned into pcDNA 6/BioEase or pENTR/D TOPO vector using a pENTR Directional TOPO cloning kit, and subcloned into pcDNA 6/BioEase or pET104 BioEase using Gateway System (Invitrogen). Sequences were confirmed by automated DNA sequencing. C-terminal His$_{6}$-tagged wild type caspase-3 was a gift from Dr. G. Salvesen (Burnham Institute) and Dr. Takahashi (Brain Science Institute). C-terminal His$_{6}$-tagged active site mutant caspase-3 was a gift from Dr. C. Clark (North Carolina State University).

Plasmids

Calcineurin B cDNA was PCR-amplified from human calcineurin B cDNA (kindly provided by Dr. Shibasaki), cloned into pENTR/D TOPO vector using a pENTR Directional TOPO cloning kit, and subcloned into pcDNA 6/BioEase or pET104 BioEase using Gateway System (Invitrogen). Sequences were confirmed by automated DNA sequencing. C-terminal His$_{6}$-tagged wild type caspase-3 was a gift from Dr. G. Salvesen (Burnham Institute) and Dr. Takahashi (Brain Science Institute). C-terminal His$_{6}$-tagged active site mutant caspase-3 was a gift from Dr. C. Clark (North Carolina State University).

Cell Culture

Human embryonic kidney 293 (HEK293), MCF-7, and PC12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 1 μl/ml amphotericin B. Jurkat cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum.

Animals

Experimental protocols involving laboratory animals were approved by the Osaka University Animal Care and Use Committee. Wistar rats pregnant for 17 days were anesthetized under ether inhalation, and rat fetuses were removed from the uteri by Cesarean section. Cortex was prepared from fetal and neonatal rats under ether inhalation.

Stable Transfection

HEK293 cells were transfected with either pcDNA 6/BioEase-GW/lacZ (control) or human calcineurin B-containing plasmid using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Clones stably expressing these constructs were then selected by growth in 2.5 μg/ml blasticidin (Invitrogen) for 3 weeks. Individual blasticidin-resistant clones were examined for expression of calcineurin B by immunoblotting with anti-calcineurin B antibody.

Subcellular Fractionation

Cytosolic extract was prepared, as described previously (6). Brain tissue or cells were washed twice with phosphate-buffered saline and suspended in 20 mM HEPES buffer (pH 7.4) containing 10 mM KCl, 1.5 mM MgCl$_{2}$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose (buffer A), and protease inhibitor mixture (Roche Applied Science) and disrupted with a Dounce homogenizer. A postnuclear supernatant was centrifuged at 10,000 × g for 20 min to obtain a pellet rich in mitochondria. The postmitochondrial supernatant was further centrifuged at 100,000 × g for 1 h to obtain the cytosolic extract.

Cell-free Assay for Cytochrome c-dependent Activation of Caspase-3

Aliquots of extracts were incubated with 2 μM horse heart cytochrome c (Sigma) and 1 mM dATP (Sigma) for 2 h at 30°C. After the incubation, cytochrome c-activated extract was incubated with 50 μM fluorescent substrate (Ac-DEVD-AMC) in 40 μl of 20 mM PIPES (pH 7.2) containing 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, and 300 mM sucrose (caspase assay buffer). Fluorescence was measured using an excitation wavelength of 380 nm and emission wavelength of 460 nm.

Purification of Caspase Activation Factor from Cytosolic Extract Prepared from Rat Cortex of Postnatal Day 7

All purification steps were carried out at 4°C. Step 1: Mono Q Chromatography—The cytosolic extract was loaded onto a Mono Q 5/5 fast protein liquid chromatography column (Amersham Biosciences) pre-equilibrated with buffer A. The column was washed with 2 column volumes of buffer A and eluted with a 20-ml linear gradient of 0–1 M NaCl. Fractions of 1 ml were collected. Aliquots were resolved by SDS-PAGE and analyzed by immunoblotting with anti-caspase-3 antibody, using enhanced chemiluminescence-based detection (Amersham Biosciences), as described previously (25).

Step 2: Gel Filtration Analysis—The pooled fractions number 6 and 7 in MonoQ were separated by a Superose 6 fast protein liquid chromatography column (Amersham Biosciences) pre-equilibrated with buffer A at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. Aliquots were analyzed as described for step 1. For calibration of the column, Amersham Biosciences high molecular mass gel filtration protein standards were used (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa).

Chemical Cross-linking

The fractions were incubated with disuccinimidyl suberate (DSS; Pierce) or dimethyl sulfoxide alone. After a 30-min incubation at room temperature, the reaction was quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 50 mM.
Activation of Procaspase-3 by Calcineurin

Cross-linked samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-caspase-3 antibody.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out by isoelectric focusing on 24-cm immobilized pH gradient strips (pH 4–7) using the IEPgopher system, followed by SDS-PAGE using Multiphor II system (Amersham Biosciences). Protein aliquots dissolved in rehydration buffer (8 M urea, 2% CHAPS, 60 mM DTT, and 0.5% immobilized pH gradient buffer) were applied to the immobilized pH gradient strips and rehydrated for 12 h. After rehydration, isoelectric focusing was performed for a total of 17,500 V-h by application of 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 2 h. Focused strips were then incubated in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, and 0.25% DTT) for 15 min followed by an additional 15-min incubation using equilibration buffer with 4.5% iodoacetamide instead of DTT. The strips were applied to 15% SDS-polyacrylamide gels (ExcelGel), and electrophoresed under standard conditions. The gel was electroblotted onto a polyvinylidene difluoride membrane using the Bio-Rad Transblot semidry blotter system or stained with silver.

Production of Recombinant Protein

BL21 (DE3) pLysS (Novagen) was transformed with pET-23b plasmid containing C-terminal His$_6$-tagged wild type or active site mutant procaspase-3 (C173S). Protein was expressed by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C and purified with cobalt-coated Sepharose beads (Clontech). The sample was equilibrated on PD-10 columns (Amersham Biosciences) with buffer A and further purified on a Mono-Q HT5/5 column driven by a fast liquid chromatography system. The Mono-Q column was equilibrated with buffer A and subsequently eluted with a gradient of 0–1 M NaCl. The peak fractions containing caspase-3 were collected and stored at −80 °C. Biotin-tagged calcineurin B was purified with streptavidin-agarose (Invitrogen).

In Vitro Cleavage of Procaspase-3 by Caspase-9 or Granzyme B

Recombinant procaspase-3 (8 nM; Calbiochem) or cytosolic extract from rat cortex of postnatal day 7 was incubated for 1 h at 37 °C in a 20 μl of caspase assay buffer in the absence or presence of recombinant active caspase-9 (20 units/ml) or recombinant granzyme B (2 μg/ml). Aliquots were resolved by SDS-PAGE and analyzed by immunoblotting with anti-caspase-3 antibody or stained with Coomassie Brilliant Blue. The cytosolic extract contains approximately the same concentration (8 nM) of procaspase-3 as the recombinant extract.

Overlay Assay Using Recombinant Caspase-3 as a Probe

The partially purified extract prepared from rat cortex was subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The membrane was then blocked for 1 h in TBST containing 5% dry milk. Recombinant procaspase-3 proteins diluted in TBST were overlaid onto the membrane and incubated for 4 h at 4 °C. After washing, the recombinant procaspase-3 was detected by the HisProbe kit (Pierce).

Tandem Mass Spectrometry

The targeted protein spot was excised from the gel and subjected to in-gel digestion with trypsin to recover the peptide. The resultant peptides were analyzed by nanoscale high performance liquid chromatography on a C18 column (0.1 × 50 nm; Michrom BioResources, Inc.) coupled to a tandem mass spectrometer (Q-ToF2; Micromass, Manchester, UK), and the data were used to search against the NCBI data base with MASCOT software (Matrix Science, London, UK) for protein identification.

Detection of Apoptosis

Cell Death Detection ELISA (Roche Applied Science) is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies against DNA and histone to detect internucleosomal fragmented DNA. The assay was performed according to the manufacturer’s instructions.

Calmodulin-Agarose Bead Pull-down Assay

The calmodulin bead assay was performed as described previously (26). Calmodulin-agarose beads (Sigma) were washed three times in preincubation buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 2 mM CaCl$_2$) to activate calmodulin. Recombinant procaspase-3 C173S mutant and calcineurin (Upstate) were then incubated with beads for 3 h at 4 °C. After washing, the beads were boiled in SDS sample buffer, and supernatants were analyzed by SDS-PAGE.

Immunodepletion of Calcineurin B

Jurkat cell extract in buffer A was incubated with anti-calcineurin B antibody for 3 h. After the incubation, extract was recovered by centrifugation. The depletion of calcineurin B was repeated three times and confirmed by immunoblotting with anti-calcineurin B antibody.

Knockdown Experiments

The 25-nucleotide modified synthetic RNAs (stealth RNAi; Invitrogen) were synthesized without overhang at the 3’ end. Sequences were as follows: sense, 5′-UUUCCAGAGAUAACUUUGCCCUAUU-3′; antisense, 5′-AAUGGCGCAAGUUAUCUUUGGAAA-3′. Stealth RNAi-negative control (Invitrogen) was used as a control. Cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

Statistical Analysis

Data are expressed as mean ± S.E. Statistical differences between groups were determined using a Tukey test after analysis of variance. $p < 0.05$ was considered significant.

RESULTS

Purification and Identification of the Caspase Activation Factor—In previous studies, we have shown that procaspase-3 exists in a high molecular weight complex in neonatal rat brain (24). During the studies, we observed that procaspase-3 in neonatal rat brain was susceptible to activation by recombinant caspase-9. As shown in Fig. 1, active caspase-9 processed only a part of the recombinant procaspase-3. However, incubation of
cytosolic extract with active caspase-9 resulted in dramatic conversion of procaspase-3 into an active form. The mixture of recombinant procaspase-3 and cytosolic extract was incubated for 1 h at 37 °C in the absence or presence of recombinant active caspase-9 or recombinant granzyme B. Aliquots were analyzed by SDS-PAGE and immunoblotting with anti-caspase-3 antibody. Procaspase-3 (32 kDa) and cleaved products (20 and 17 kDa) are indicated.

To purify the caspase activation factor in the high molecular weight complex, we performed a cell-free assay for caspase activation using cytosolic extract of PC12 cells. As a first step in the purification, crude cytosolic extract prepared from rat cortex of postnatal day 7 was applied onto a Mono Q column and eluted with a linear NaCl gradient. As shown in Fig. 2, fractions number 6 and 7 showed high caspase-3-activating activity. Although these fractions contained an additional amount of pro-caspase-3, compared with the extract of PC12 cells alone, the resulting caspase-3 activity was significantly higher than that of the extract of PC12 cells in which a corresponding amount of recombinant procaspase-3 was added. These fractions were suggested to include the factor that activates caspase-3 activity. Pooled fractions number 6 and 7 were subjected to further purification by gel filtration chromatography. As shown in Fig. 3, fraction 15 showed high caspase-3-activating activity.

In order to clarify the components of the high molecular weight complex, we chemically modified the fraction 15 with DSS. As shown in Fig. 4, DSS produced procaspase-3-reactive bands with molecular masses of 32, 64, 85, and 170 kDa. Although the band of 64 kDa is considered as a dimer of pro-caspase-3, the band of 85 kDa is not a multiplied complex of procaspase-3, suggesting that it may contain another protein of 20 kDa. By immunoblotting with anti-cleaved caspase-3 antibody, we confirmed that active caspase-3 is not a component of the complex. Therefore, the complex consisted of a dimer of procaspase-3 and an unidentified 20-kDa protein. We decided to identify this 20-kDa protein.

To identify the procaspase-3-interacting protein, we used a two-dimensional gel electrophoresis far Western immunoblot approach. Initially, we attempted to generate recombinant wild type procaspase-3 as a probe. As reported previously, wild type procaspase-3 underwent proteolysis during purification, and the purified preparations contained mainly cleaved forms of procaspase-3 (data not shown). Consequently, we generated a recombinant active site mutant of procaspase-3 (C173S) to prevent its cleavage. The partially purified fraction of the gel filtration was separated by two-dimensional gel electrophoresis, transferred to a membrane, and probed with the recombinant procaspase-3. Among the protein spots revealed by silver stain (Fig. 6A), only one spot was labeled with the recombinant caspase-3 (Fig. 6A).
The spot was excised from the gel and subjected to in-gel digestion with trypsin. The eluted peptides were sequenced using mass spectrometry, and the obtained sequence, VIDIFDT-DGNGEVDFK, matched the sequence of rat calcineurin B (Fig. 6B). To confirm the direct binding of calcineurin to procaspase-3, a pull-down assay was performed using purified recombinant proteins. Recombinant procaspase-3 was pulled down in the presence of calcineurin with calmodulin-agarose beads (Fig. 7).

Expression of Calcineurin B Potentiates Apoptosis and Caspase-3 Activation Induced by TNF-α/CHX—We generated HEK293 clones stably expressing biotin-tagged calcineurin B. HEK293 cells were transfected with a control vector or a calcineurin B-containing vector. The control vector did not modify calcineurin B expression as compared with the parental HEK293 cells. The expression of calcineurin B was confirmed by immunoblotting (Fig. 8A). To examine apoptosis in the stable cell line, HEK293 cells were treated with TNF-α and CHX for 24 h. Apoptosis was assessed using cell death detection ELISA. As shown in Fig. 8B, calcineurin B enhanced apoptosis induced by TNF-α and CHX. In addition, calcineurin B enhanced the activation of caspase-3 induced by TNF-α and CHX (Fig. 9A). Extracts from cells stably transfected with a control vector or a calcineurin B-containing vector were incubated with or without cytochrome c. Overexpression of calcineurin B increased cytochrome c-dependent caspase-3 activation (Fig. 9B). When overexpressed, calcineurin B may make a complex with the preexisting endogenous calcineurin A, and this complex may account for the effect obtained with overexpression of calcineurin B. We examined the effect of calcineurin inhibitor tacrolimus. As shown in Fig. 10, tacrolimus had no effect on calcineurin B-induced potentiation of apoptosis. This result suggests that the effect of calcineurin B on apoptosis is independent of its phosphatase activity.

Depletion of Calcineurin B Inhibits Caspase-3 Activation—In order to examine the effect of endogenous calcineurin B, we employed the Jurkat T-cell line that is a commonly used and established cellular model system for the elucidation of apoptotic pathways. Jurkat cell extracts were immunodepleted of calcineurin B using anti-calcineurin B antibody. Immuno depletion resulted in a relative decrease in calcineurin B content (Fig. 11A) and a corresponding reduction in cytochrome c-induced caspase-3 activation (Fig. 11B). To assess the role of calcineurin B in cytochrome c-induced caspase-3 activation, we added purified calcineurin B to the calcineurin B-depleted extracts (Fig. 11C). The addition of calcineurin B potentiated cytochrome c-induced caspase-3 activation in a dose-dependent manner (Fig. 11D).

Calcineurin B Small Interference RNA (siRNA) Inhibits Apoptosis Induced by TNF-α/CHX—We also tested the effect of siRNA knockdown of calcineurin B on apoptosis in HEK293 and MCF-7 cells. Both in HEK293 and MCF-7 cells, the calcineurin B level in siRNA-treated cells was reduced to 20% of normal level (Fig. 12A). In HEK293 cells, treatment with siRNA against calcineurin B resulted in a significant reduction of apoptosis induced by TNF-α and CHX (Fig. 12B). A protective effect of siRNA was also observed when we used staurosporine instead of TNF-α and CHX (data not shown). It has been demonstrated that the MCF-7 cell is devoid of caspase-3 due to the functional deletion of the caspase-3 gene (27). Several reports have shown that although caspase-3 is required for some of the typical morphological changes of cells undergoing apoptosis, DNA fragmentation occurs in the absence of caspase-3 in MCF-7 cells. In MCF-7 cells, the calcineurin B siRNA had no significant effect on apoptosis induced by TNF-α and CHX.
In addition, the calcineurin B siRNA had no significant effect on cytochrome c-dependent caspase-9 activation in MCF-7 extract (Fig. 12D). These observations, together with the results from immunodepletion experiments, indicate that endogenous calcineurin B may function as a regulator of caspase-3 activation.

**DISCUSSION**

In this study, we report the purification of a novel procaspase-3-binding protein in rat brain. As shown in Fig. 1, the cytosolic extract from rat embryonic brain promoted the activation of procaspase-3 by caspase-9. Together with the previous findings that procaspase-3 exists in a high molecular weight complex in neonatal rat brain (24), these data indicate the existence of a procaspase-3 activation mechanism in neonatal brain.

First, we tried to purify and identify the activation factor, using ant-caspase-3 as an indicator, with various chromatographic procedures (Figs. 2 and 3). In order to detect the 20-kDa factor in the further purification, we developed and adopted the inactive recombinant procaspase-3 because of avoidance of autodigestion (Fig. 5). Based on the observation that procaspase-3 interacts with an unidentified 20-kDa protein (Fig. 4), we have performed a two-dimensional gel electrophoresis far Western immunoblot (Fig. 6). Tandem mass spectrometry identified this protein as calcineurin B. We confirmed that calcineurin B possesses an ability to interact with procaspase-3 (Fig. 7).

Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase that consists of a catalytic A subunit and a regulatory B subunit (28). Calcineurin plays an important role in the coupling of Ca$^{2+}$/H$^{+}$ signals to cellular responses (29). A major consequence of Ca$^{2+}$ mobilization is activation of the transcription factor NFAT (30). NFAT is a family of highly phosphorylated proteins residing in the cytoplasm of resting cells. When cells are activated, NFAT is dephosphorylated by calcineurin, translocates to the nucleus, and becomes transcriptionally active (31). Among the diverse functions of calcineurin, its role in the immune system is well understood. Calcineurin is the molecular target of immunosuppressive agents, cyclosporin A and tacrolimus (32–37). It has also been reported that calcineurin is a critical effector of cell death. In BHK-21 cells, overexpression of active calcineurin induces apoptosis (38).

Although the downstream target of calcineurin is unclear, a recent study provided evidence that calcineurin is responsible for BAD dephosphorylation and subsequent cell death of primary hippocampal neurons exposed to glutamate (39, 40). Therefore, so far, phosphatase activity of calcineurin has been responsible for the regulatory role in cell death.

Cancer cells survive despite initiation of upstream apoptotic responses. Therefore, the elucidation of the mechanism of procaspase-3 activation and its regulation downstream of cytochrome c has clinical importance. It has been reported that heat shock proteins accelerate activation of procaspase-3 (22, 23). In addition, it is interesting to note that an autoinhibitory domain exists in procaspase-3 (41). Since
procaspase-3 has been reported to be particularly resistant to processing compared with other caspases (41), the formation of the complex may explain the susceptibility to the activation of caspase-3 and subsequent apoptosis in the developing brain.

To clarify the participation of calcineurin B in apoptosis, we investigated in vitro and in vivo effects of overexpression and depletion of calcineurin B in HEK293 cells and Jurkat cells. The calcineurin B transfection significantly potentiated the susceptibility to apoptosis induced by TNF-α and CHX (Fig. 8).
through the activation of caspase-3 (Fig. 9). In addition, cytochrome c-induced activation of caspase-3 in the calcineurin B-depleted cell extract was reduced (Fig. 11), and knockdown of calcineurin B inhibited TNF-α-induced apoptosis (Fig. 12). A regulatory role of calcineurin B for phosphatase activity of calcineurin A has been well established (42, 43). Tacrolimus binds to immunophilin and inhibits phosphatase activity of calcineurin (32–37). Tacrolimus failed to affect TNF-α-induced apoptosis in calcineurin B-transfected cells (Fig. 10), suggesting that phosphatase activity may not be involved in the apoptosis-promoting ability of calcineurin B.
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These results suggest that calcineurin B may possess a novel function to participate in the regulation of apoptosis, in addition to its well known ability to regulate calcineurin A. Furthermore, calcineurin B itself has no effect on apoptosis but promotes apoptotic signal transduction system through the presentation of scaffold for the activation of caspase-3 by caspase-9. Therefore, calcineurin B may be useful for therapies to induce and promote apoptosis.

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