Stress-induced Apoptosis*

Nucling Recruits Apaf-1/Pro-caspase-9 Complex for the Induction of Stress-induced Apoptosis*

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Nucling is a novel protein isolated from murine embryonal carcinoma cells with an up-regulated expression during cardiac muscle differentiation. We show here that Nucling was up-regulated by proapoptotic stimuli and important for the induction of apoptosis after cytotoxic stress. We further demonstrated that overexpressed Nucling was able to induce apoptosis. In Nuclinge deficient cells, the expression levels of Apaf-1 and cytochrome c, which are the major components of an apoptosis-promoting complex named apoptosome, were both down-regulated under cellular stress. A deficiency of Nucling also conferred resistance to apoptotic stress on the cell. After UV irradiation, Nucling was shown to reside in an Apaf-1/pro-caspase-9 complex, suggesting that Nucling might be a key molecule for the formation and maintenance of this complex. Nucling induced translocation of Apaf-1 to the nucleus, thereby distributing the Nucling/Apaf-1/pro-caspase-9 complex to the nuclear fraction. These findings suggest that Nucling recruits and transports the apoptosome complex during stress-induced apoptosis.

Cell death is classified into two major morphologically and biochemically distinct modes, necrosis and apoptosis. Necrosis is characterized by swelling of organelles and cells, followed by lysis of the plasma membrane and random DNA degradation. In contrast, apoptosis is a process that is characterized by cell shrinkage, plasma membrane blebbing, nuclear condensation, and endonucleolytic cleavage of DNA into fragments of oligonucleosomal length and is a fundamental and indispensable process during normal embryonic development, tissue homeostasis, and regulation of the immune system (1–3). In addition, environmental stressors such as heat shock, radiation, chemical agents, and oxidative stress can also induce apoptosis. These proapoptotic stimuli bring about organellar stress, affecting the nucleus, peroxisome, lysosome, or Golgi apparatus. Most of the apoptosis-inducing signals from these organelles converge at mitochondria. Mitochondria release proteins that promote cell death after cellular stress (4). One of these proteins is cytochrome c, which forms a complex with the cytoplasmic protein Apaf-1 and pro-caspase-9, leading to the activation of caspase-9. Caspase-9, in turn, activates caspase-3, the protease that cleaves the majority of caspase substrates during apoptosis. Mitochondria also release apoptosis-inducing factor (AIF) and endonuclease G, which appear to kill cells independently of caspases. Therefore, mitochondria are thought to be a central regulatory element in stress-induced apoptosis (5).

Nucling was originally isolated from murine embryonal carcinoma cells as a protein, the expression of which was up-regulated during cardiac muscle differentiation (6). A bovine homolog of Nucling, named βCAP73, was isolated and characterized as a novel regulator of β-actin assembly (7, 8). Recently, we reported that Nucling down-regulates expression of the antiapoptotic molecule, galectin-3, through interference with nuclear factor-κB (NF-κB) signaling (9). It has been reported that galectin-3 is translocated to the perinuclear membranes after exposure to a variety of apoptotic stimuli and becomes abundant in mitochondria, where it prevents mitochondrial damage and cytochrome c release (10). These findings led us to investigate whether Nucling is involved in any of the apoptotic signaling pathways. To address this issue, we performed molecular biological analyses, including gene knock-out experiments.

In this report, we show that Nucling is a regulatory molecule for stress-induced apoptosis, which interacts with the Apaf-1/pro-caspase-9 complex, thereby acting as a stabilizer for the apoptosome. Nucling was shown to be able to induce apoptosis in mammalian cells and could promote the caspase cascade. Nucling−/− cells showed resistance to cellular stress. Up-regulation of Apaf-1, release of cytochrome c, and activation of caspase-9 induced by cytotoxic stress were not observed in Nucling−/− cells. Two-dimensional native/denaturing-PAGE analysis revealed that Nucling assembles with the Apaf-1/pro-caspase-9 complex.

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†† The abbreviations used are: AIF, apoptosis-inducing factor; NF-κB, nuclear factor-κB; MEF, mouse embryonal fibroblast; PI, propidium iodide; TUNEL, TdT-mediated DUTP-biotin nick end labeling; LDH, lactate dehydrogenase; Nucl.mid, middle portion of Nucl; RT-PCR, reverse transcription-polymerase chain reaction; BAP, bacterial alkaline phosphatase; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; zQDMID-fmk, benzoyloxycarbonyl-Asp-Glu-Met-Asp-fluoromethyl ketone; TNF, tumor necrosis factor; CHX, cycloheximide; WT, wild type.
caspase-9 complex in vivo. These findings suggest that Nuclng acts as a regulatory factor for stress-induced apoptosis, sustaining the expression level of Apaf-1 by interacting with the Apaf-1/c-caspase-9 complex. Moreover, this association complements the expression of Nuclng, Apaf-1, and c-caspase-9 was observed in both cytosolic and nuclear fractions. Furthermore, we confirmed that Nuclng was required for the translocation of Apaf-1 to the nucleus after proapoptotic stress, suggesting that Nuclng is a transporter of the Apaf-1/c-caspase-9 complex to the nucleus.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—COS7 cells, HeLa cells, and mouse embryonic fibroblast (MEF) cells were used in this study. Cells were maintained in DMEM with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 5 μM mercaptoethanol, and 10% (v/v) fetal calf serum. They were cultured in 10 ml of medium in 95-mm plastic tissue culture plates at 37°C in an atmosphere of 5% CO2/95% air in a humidified incubator. For routine propagation, cultures were split, and the growth medium was replenished every three to four days.

**Western Blot Analysis**—Cellular extracts were prepared as described previously (6). Antibodies reactive to cytochrome c (BD Biosciences), Apaf-1 (Chemicon), caspase-9 (Cell Signaling), caspase-3 (BD Biosciences), β-actin (Sigma-Aldrich), and the middle portion of Nuclng (Nuclmid) (6) were used in this study. Western blot analysis was carried out according to standard procedures using an ECL detection kit (Amersham Biosciences) or AP detection kit (Roche). Quantification was achieved by comparing densitometric scanning readings using NIH-Image v1.63 software; numbers (arbitrary units) represent values corrected for loading with the data reprobed by β-actin.

**Northern Blot Analysis**—After treatment, COS7 cells were washed with PBS and pelleted by centrifugation. Total RNA was isolated from cells using ISOGEN as described by the manufacturer (Nippon Gene, Toyama, Japan). RNA was fractionated on 2.2 M formaldehyde/1% agarose gels and transferred overnight onto Hybond N nylon membranes (Amersham Biosciences) in 10× SSC. The RNA was cross-linked to the membrane using a UV cross-linker (Amersham Biosciences) before hybridization. A specific probe was generated by labeling the cloned cDNA fragment of full-length Nuclng with [α-32P]dCTP (NEN, Boston, MA) using Ready-To-Go DNA Labeling Beads (dCTP) (Amersham Biosciences). After overnight hybridization at 42°C, the filters were washed once in 2× SSC for 10 min (23°C) and twice in 0.1× SSC for 15 min (68°C), covered in plastic wrap, and exposed to Kodak X-Omat AP film at −70°C for 3–24 h.

**Generation of Nuclng−/− Mice**—The genomic DNA containing the Nuclng gene was isolated from a 129/Sv mouse genomic library. The targeting vector was constructed by inserting a PGK-neo-poly(A) cassette into a HindIII site of the exon containing the leucine zipper motif region (6) of the Nuclng gene. The targeting vector thus contained 0.5- and 5.4-kb regions of homology in the 5′ and 3′ region of the neomycin-resistance marker, respectively. The maintenance, transfection, and selection of embryonic stem cells were performed as described (11). The mutant embryonic stem cells were microinjected into C57BL/6J blastocysts, and the resulting male chimeras were mated with female C57BL/6 mice. Heterozygous offspring were intercrossed to produce homozygous mutant animals. All mice were maintained in a specific pathogen-free animal facility.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Total RNA was purified from COS7 cells using ISOGEN. RT was performed using 2 μg of total RNA (at 42°C for 2 h) in a 20-μl reaction volume containing oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). The PCR primers, designed based on the published sequence of cytochrome c, and Nuclng were 5′-CGAATTCTTTTGAAGTGTGTTGA- A-3′ (cytochrome c, sense), 5′-GTGGAATTCCTAGAGCGCTTTTT- TAAAG-3′ (cytochrome c, antisense), 5′-TGTACCCCGAGGCGGAAA- GTTACC-3′ (Nuclng, sense), and 5′-GGTGCCTTTGGAGCGGAGG- AAGTGG-3′ (Nuclng, antisense). The primers 1 and 14 designed by Honarpour et al. (12) were used for the Apaf-1 message. For PCR, 2 μl of cDNA was used in a 50-μl reaction mixture containing 0.5 mM primers, deoxynucleotide triphosphates, and TaqDNA polymerase, using the cycling profile for 45 of a 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C for 24 cycles for cytochrome c and 28 cycles for Apaf-1 with a final extension at 72°C for 10 min. The cycling profile for Nuclng was 1 min at 95°C, 1 min at 56°C, and 2 min at 72°C for 30 cycles with a final extension at 72°C for 10 min. PCR products were analyzed on 2% agarose gel. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (6) or β-actin was used as a control.

**Two-dimensional Native/Denaturing-PAGE Analysis of Protein Complexes**—Wild-type or Nuclng−/− MEF cell lysates were fractionated into cytosol or nuclear fraction as described before (6). The fractions were analyzed directly by native-PAGE (13). In short, cell lysates were resolved onto 2% polyacrylamide gels and transferred overnight onto Hybond N nylon membranes (Millipore, Bedford, MA) in the same buffer using the semi-dry blotting technique. Immunodetection was carried out according to standard procedures and was visualized by the ECL method (Amersham Biosciences). For two-dimensional gel analysis, individual lanes were cut out from the first-dimension native gel and layered on top of a 15–25% gradient resolving gel, and a 7% stacking gel was poured over and around the native gel slice.

**RESULTS**

**Nuclng Is a Potent Promoter of Apoptosis**—During the course of our previous study to identify Nuclng (6), we had been examining the functional role of this molecule and noticed that the transfection efficiency of the Nuclng-expressing plasmid into mammalian culture cells was very low. In addition, the overexpressed Nuclng brought about nuclear deformation or fragmentation (data not shown). These findings led us to suspect that Nuclng might be able to induce apoptosis. To investigate this possibility, we performed PI staining and TUNEL staining on transfected cells.

PI has been used as a marker of cell damage. We first checked whether the nuclei of cells could be stained with PI without permeabilization by immunofluorescence microscopy. An ~10-fold excess of cells transfected with pFlag-Nuclng...
were stained with PI compared with control cells transfected with pFlag-BAP (data not shown). In addition, we observed a strong correlation between the Flag-Nucling-expressing cells (Fig. 1Aa) and PI staining (red in 1B and yellow in 1C), whereas no correlation was found between Flag-BAP-expressing cells (control; Fig. 1Ad) and PI staining (red in 1B and yellow in 1C). TUNEL assay also supported the possibility of apoptosis. We found a strong correlation between the Flag-Nucling-expressing cells (Fig. 1Ah) and TUNEL-positive cells (Fig. 1Ag and Ai), whereas no correlation was found between Flag-BAP-expressing cells (control; Fig. 1Ak and Al) and TUNEL-positive cells (Fig. 1Aj and Al). To confirm these results concerning cell damage, we investigated the effect of Nucling on cell survival in COS7 cells detected using the transient transfection assay with the LDH release assay. Transfection of the pFlag-Nucling-expressing plasmid in COS7 cells was performed. The LDH release activity in the culture medium was calculated every 24 h for 2 days after transfection. Transfection of pFlag-Nucling led to an increase in LDH activity compared with that of the pFlag-vector control (mock) at 24 and 48 h after transfection (Fig. 1B, columns). At 48 h, an ~20% increase in LDH activity was observed in Nucling-transfected cells compared with mock-transfected cells. This percentage was in good accord with the estimated transfection efficiency (~20%, data not shown) of the Nucling expression vector. These results indicate that Nucling possesses an intrinsic cell death-promoting activity. To investigate whether the caspase system is important for the activity of Nucling promoting cell death, we performed a zVAD-fmk inhibition assay. Cell death-inducing activity of the Nucling protein was clearly reduced by zVAD-fmk, a pan-caspase inhibitor (Fig. 1B, □). In addition, the zDQMD-fmk (an inhibitor specific for caspase-3 and caspase-6) treatment assay for the Nucling-overexpressing cells revealed that Nucling can promote cell death by activating caspase-3 or caspase-6, because this treatment clearly suppressed the cell death-inducing activity of Nucling (Fig. 1C). These results suggest that Nu-
Cling may be a member of the caspase signaling pathways. Proapoptotic Stimuli Up-regulate Cling Expression—To assess whether Cling is a part of the apoptosis signaling pathways, we investigated whether endogenous Cling expression was regulated by several proapoptotic stimuli. Northern blot analyses revealed that Cling expression was induced in COS7 cells (lane 3) and HeLa cells (data not shown) by tumor necrosis factor-α (TNF-α)/cycloheximide (CHX) (lane 3). Untreated COS7 cells (lane 1) were also prepared as a negative control. Aliquots of 20 μg of total RNA were loaded in each lane and separated by agarose gel electrophoresis. The integrity of the RNA loading was assessed by ethidium bromide staining of the 28 S and 18 S rRNA bands (lower panel). B, RT-PCR. RNA was prepared from NIH3T3 cells treated with proapoptotic stress including Adriamycin (0.5 μM) treatment for 6, 12, and 24 h or heat shock (1-h incubation at 45 °C) treatment, followed by incubation for 6, 12, or 24 h. Experiments were repeated two or three times with similar results. B, confocal images of dual immunofluorescence staining assay using the TUNEL assay system (green) and Nucl.mid antibody (red) in COS7 cells. Most of the TUNEL-positive (green) cells were stained with Nucl.mid antibody (red) as shown in TNF-α (20 ng/ml)/CHX (1 μg/ml) treated cells or H2O2 (1 mM) treated cells. Bar, 10 μm. C, H2O2 up-regulated Cling expression. Dot-spot staining in the nucleus was observed in cells at 16 h after trypsinization (0 μM). Moderate stress caused by 0.5 mM H2O2 induced Nucl mid antibody staining (middle panel) in the nucleus. Some cells were stained diffusively in the nucleus (arrowhead). Severe stress evoked by 1 mM H2O2 induced Nucl mid antibody staining (right panel) more effectively than moderate stress (middle panel). Nuclei were stained diffusively in many cells. Some of the cells were stained ubiquitously in the nucleus and cytoplasm (arrow). Bar, 10 μm.
that endogenous Nucling is induced by apoptotic stress in a dose-dependent manner.

**Nucling Is Required for UV Irradiation-induced Apoptosis**—To further address the issue of the physiological function of Nucling in vivo, we next generated Nucling−/− mutant mice. We constructed a targeting vector designed to insert the neomycin-resistance cassette into the exon encoding the LZ motif (Fig. 3A). Germline transmission was achieved from two independent clones and confirmed by Southern blot analysis (Fig. 3B). Both heterozygous and homozygous Nucling−/− mice are viable and fertile. Northern blot analysis of total RNA extracted from adult (8-week) wild-type (WT), heterozygous, and homozygous hearts and skeletal muscle using the XbaI fragment of Nucling cDNA as a probe confirmed the absence of
Nucling mRNA transcripts in homozygous-deficient mice (Fig. 3C). Thus, our targeting strategy resulted in a null allele for the Nucling gene.

To obtain direct evidence that Nucling deficiency leads to defects in the regulatory mechanism for stress-induced apoptosis, we investigated the cell-death response to UV irradiation-induced cellular stress. Exposure to excessive UV irradiation is known to cause apoptosis in murine fibroblasts (14). In addition, we further reported that Nucling down-regulated the anti-apoptotic factor, galectin-3, via the modification of NF-κB activation (9). The activation of NF-κB is known to be induced by UV irradiation, followed by the up-regulation of galectin-3 transcription. At first, we prepared MEFs from WT or Nucling−/− embryos. Western blot analysis was performed to check the expression of Nucling in WT MEFs. We could detect a distinct Nucling band in WT MEFs but not in Nucling−/− MEFs (Fig. 3D). Next, we investigated the reactivity of Nucling−/− MEFs to a proapoptotic stress using UV irradiation.

UV irradiation induced an increase in cell death in WT MEFs as compared with Nucling−/− MEFs (Fig. 4). Although the total amount of LDH in Nucling−/− MEFs was less than that in WT MEFs, significant levels of cell killing were observed, arguing that UV irradiation-induced apoptosis has a partial Nucling dependence. Therefore, we assumed that Nucling regulates one or some of the many apoptotic pathways induced by cytotoxic stress from UV irradiation.

**Nucling May Play a Critical Role in the Expression of Apoptosome-related Molecules**—To elucidate the molecular mechanism underlying the apoptosis-promoting activity of Nucling, we investigated expression levels or activation patterns of several candidate molecules related to stress-induced apoptosis. We compared first the expression levels of Apaf-1 between WT and Nucling−/− mice in MEFs treated with H2O2 (Fig. 5A) or UV irradiation (Fig. 5B). At first, we determined whether any defect occurred in the activation of caspase-9 in Nucling−/− MEFs. Immunoblot analysis revealed that not only the proenzymatic form of caspase-9 (p37) does exist in WT MEFs, but no processing was observed in Nucling−/− MEFs under normal culture conditions (Fig. 5, A and B, lanes 1 and 2). In addition, this tendency was also observed in response to the apoptotic stimulus of H2O2 (Fig. 5A, lanes 3 and 4) or UV irradiation (Fig. 5B, lanes 5 and 6). The apoptotic pathway mediated by caspase-9 is initiated by the release of cytochrome c from mitochondria (15, 16). Therefore, we examined the expression level of cytochrome c in these MEFs. Exposure of WT MEFs to H2O2 caused a large increase in cytoplasmic cytochrome c (Fig. 5A, lane 3). The same result was observed using UV irradiation. In contrast, the apoptotic stress of H2O2 or UV irradiation did not cause cytochrome c up-regulation in Nucling−/− MEFs. In addition, the expression level of cytochrome c in whole-cell lysate from Nucling−/− MEFs was also reduced after H2O2 treatment or UV irradiation (Fig. 5, A and B). Western blot analysis revealed that the total level of cytochrome c in the normal culture without proapoptotic stress was almost the same between WT and Nucling−/− MEFs (Fig. 5A, lanes 1 and 2). In contrast, cytochrome c expression was remarkably down-regulated in Nucling−/− MEFs compared with WT MEFs after the proapoptotic stress (Fig. 5, lanes 3 and 4 in A, lanes 3–6 in B). These results indicate that Nucling is essential for the down-regulation of cytochrome c in response to apoptotic stimuli but not for the release of cytochrome c from mitochondria. Western blot analysis also revealed that Apaf-1 was strikingly down-regulated in its expression in Nucling−/− MEFs after proapoptotic stress (H2O2 in Fig. 5A and UV irradiation in Fig. 5B). In contrast, the expression level of AIF, a mitochondrial apoptosis-inducing factor, was the same in both WT and Nucling−/− MEFs (Fig. 5B). Active caspase-9 (p37) was not detectable in Nucling−/− MEFs (Fig. 5A, and B). Pro-caspase-3 (apoptosis executioner regulated by caspase-9) remained in its inactive form in Nucling−/− MEFs (Fig. 5A, lane 4). This observation can be explained by the absence of p37 in Nucling−/− mice. These results strongly suggest that Nucling may be an apoptosis-promoting factor specifically regulating the Apaf-1/cytochrome c/caspase-9 apoptosome pathway after cellular stress. Semiquantitative RT-PCR analysis revealed that the transcriptional levels of both cytochrome c and Apaf-1 expression were not decreased in Nucling−/− MEFs under proapoptotic conditions as shown in Fig. 5C. Both the 0.3-kb fragment of cytochrome c and the 0.5-kb fragment of Apaf-1 were amplified from the RNAs of WT and Nucling−/− MEFs. We could not observe any obvious change in the amplification reactions between these MEFs in response to the proapoptotic stimuli. Consequently, we postulated that the down-regulation of cytochrome c in Nucling−/− MEFs comes from the post-transcriptional regulation of the apoptosome molecules, Apaf-1 and cytochrome c, after proapoptotic stress. Therefore, we focused on investigating the regulatory role of Nucling for the apoptosome components in terms of their interactions at the protein level.

**Nucling Plays a Critical Role in the Nuclear Translocation of Apaf-1 after Proapoptotic Stress**—To confirm whether Nucling intrinsically up-regulates Apaf-1, a double immunostaining assay was performed after transfection of the Nucling expression vector into COS7 cells. The intensity of red-stained endogenous Apaf-1 was up-regulated in most of the Nucling-overexpressing cells (green in Fig. 6A). Very interestingly, the staining pattern of Apaf-1 in the Nucling-overexpressing cells was different from that of nontransfected cells. Ectopic expression of Nucling induced translocation of Apaf-1 from cytoplasm to nucleus (Fig. 6A). To investigate whether Nucling physiologically regulates the nuclear translocation of Apaf-1, we checked the expression profiles of Apaf-1 in WT and Nucling−/− MEFs after UV irradiation (Fig. 6B). We first confirmed the nuclear redistribution of Apaf-1 in WT MEFs after proapoptotic stress as reported previously (17). We found that cytochrome c redistributes 8 h...
after UV irradiation, forming a ring structure (indicated by an arrow in Fig. 6Bg). In addition, we detected a diffuse cytochrome c pattern in the nucleus (arrowhead in Fig. 6Bh) at the time point when >40% of cells were clearly apoptotic (data not shown). In contrast, Apaf-1 was translocated to the nucleus in most of the cytochrome c redistributed cells (yellow arrows in Fig. 6Bh). In Nuclong−/− MEFS, up-regulation of cytochrome c in the cytoplasm was observed as well (arrow in Fig. 6Bj). However, nuclear localization of Apaf-1 in these cells was not observed, and the expression pattern of Apaf-1 mostly overlapped that of cytochrome c (arrow in Fig. 6Bh). These results strongly suggest that Nuclong might directly regulate the redistribution of Apaf-1 into the nucleus after proapoptotic stress.

Nuclong Assembles with Apaf-1/Pro-caspase-9 Complex in Vivo—On the basis of the finding that Nuclong regulated the expression pattern of apoptosome molecules at the protein level, we focused on whether Nuclong directly interacts with Apaf-1, caspase-9, or cytochrome c. First, the interaction between Nuclong and Apaf-1 was investigated with coimmunoprecipitation assays. As shown in Fig. 7A, endogenous Nuclong in COS7 cells was coimmunoprecipitated with Flag-tagged Apaf-1 by anti-Flag antibody. To visualize a protein complex containing Nuclong or Apaf-1 directly, the cytosol fraction (Fig. 7B) or nuclear fraction (data not shown) was separated under nondenaturing conditions. Immunoblot of these native gels containing Nuclong or Apaf-1 directly, the cytosol fraction (Fig. 7Bd) was also detected.
Nucling Regulates Stress-induced Apoptosis

Mitochondria play a key role in the commitment of cells to apoptosis through the release of cytochrome c and many apoptosis-inducing factors from the intermembrane space into the cytosol (18–25). Several proapoptotic stimuli induced endogenous Nucling expression in the nucleus, followed by the cytoplasm (Fig. 2). We also observed that Nucling was a crucial molecule for Apaf-1, cytochrome c up-regulation, and caspase-9 activation but not for AIF induction after exposure to a proapoptotic stimulus such as UV irradiation or H2O2 stress. These findings indicate that Nucling acts as a component of the mitochondrial apoptotic pathways, especially of the cytochrome c/Apaf-1/caspase-9 apoptosome pathway. Furthermore, a lack of Nucling expression conferred on MEF cells resistance to apoptosis after cytotoxic stress from UV irradiation (Fig. 4).

It was reported that low levels of or a deficiency in Apaf-1 protein can determine sensitivity to apoptosis downstream of mitochondrial events, suggesting that regulation of Apaf-1 may be important for apoptotic processes (26, 27). Our findings directly show the presence of a novel regulatory mechanism for Apaf-1 expression at the protein level. We confirmed that the inhibition of cytochrome c release in Nucling−/− cells comes from the post-translational down-regulation of the apoptosome molecules, Apaf-1 and cytochrome c, after a proapoptotic stress (Fig. 5). During stress-induced apoptosis, caspase activation requires a large number of post-translational events, including translocation to other organelles (28). This is also the case in the translocation of caspase-9 or Apaf-1 to the nucleus (17, 29).

Here we propose that Nucling may be a key molecule for the retention of the caspase-9/Apaf-1 complex, its translocation to the nucleus, and its activation. We also confirmed that endogenous Nucling assemblies with the Apaf-1/pro-caspase-9 complex in vivo in both the nuclear and the cytoplasmic fractions (Fig. 7). The affinity of this interaction may be weak, because a large amount of free Apaf-1 was detected in Fig. 7C, lane e. In addition, Nucling itself distributes actively to the perinucleus (6). We also found that Nucling is essential for the redistribution of Apaf-1 into the nucleus (Fig. 6). On the basis of these observations, Nucling is a strong candidate for the shuttle molecule in the translocation of caspase-9/Apaf-1 to the nucleus after stress-induced apoptosis.

It is well known that proapoptotic stimuli trigger the release of cytochrome c from mitochondria, which forms the complex with Apaf-1. In this context, the two-dimensional PAGE analysis, as shown in Fig. 7C, revealed that an Apaf-1/cytochrome c complex was present in the cytosol as an ~55-kDa protein assembly. On the other hand, cytochrome c was not detected in the nuclear fraction in the same analysis. This result may

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indicate that cytochrome c is released from Apaf-1 at the time of nuclear translocation.

As described previously, Nucling$^{-/-}$ mice displayed frequent inflammatory lesions (9) but no other defects similar to those of Apaf-1$^{-/-}$ or caspase-9$^{-/-}$ mice, including forebrain hyperplasia (11, 30, 31). Although this phenotypic discrepancy might come from the existence of unknown redundant molecules or pathways, and Nucling may not be essential for apoptosis during neural development, we observed distinct differences between WT and Nucling$^{-/-}$ mice concerning the apoptotic...
responsiveness under cellular stress. In particular, the expression levels of Apaf-1, cytochrome c, and caspase-9 in MEF cells under several forms of cellular stress differed strikingly between WT and Nuclng−/− strains. In fact, down-regulation of Apaf-1 was also observed in several Nuclng−/− tissues including kidney, spleen, and lung but not in brain (data not shown). There might be an alternative molecule(s) in place of Nuclng in the brain or neural development. There might also be a distinct signal transduction pathway for stress-induced apoptosis, different from that for developmental apoptosis. Or other apoptosis-independent mitochondrial apoptosis-inducing factors, such as AIF or endonuclease G, may be prominent in the development of Nuclng−/− mice. Actually, it has been reported that there must be an Apaf-1-independent pathway for apoptosis triggered by cytotoxic stress (32–34). Our findings may also support the hypothesis that the “regulation of Apaf-1 expression may be a regulatory mechanism developed in postmitotic cells to prevent an irreversible commitment to die after the release of cytochrome c”, proposed by Sanchis et al. (34) recently.

We reported previously that Nuclng negatively regulated the antiapoptotic molecule galectin-3 and NF-κB activation (9). NF-κB is known to be most commonly involved in suppressing apoptosis by transactivating the expression of antiapoptotic genes (35). From these findings, we concluded that a stress-induced factor, Nuclng, promotes apoptosis by regulating three pathways, apoptosis upstream regulation, galectin-3 down-regulation, and NF-κB inactivation.

This report shows that Nuclng is the regulator of Apaf-1 expression and plays an important role in the regulation of stress-induced apoptosis.

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