Prevention of Apoptosis of Mammalian Cells by the CED-3-Cleaved Form of CED-9*

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Summary. CED-9 prevents apoptosis in embryonic cells of Caenorhabditis elegans but not in mammalian cells. We show here that the prevention of apoptosis in mammalian cells requires a CED-3-cleaved form (68-280) of CED-9 which is localized in the inner mitochondrial membrane. The viability of PC12 and HeLa cells was significantly increased after death stimuli when truncated CED-9 was expressed in these cells but full-length CED-9 did not. The truncated CED-9 expressed in these cells was largely localized to the inner mitochondrial and the endoplasmic reticulum membranes, whereas full-length CED-9 was detected mainly in endoplasmic reticulum fractions. Moreover, truncated CED-9 in purified mitochondria was resistant to trypsin digestion, but full-length CED-9 was not. These results suggest that the CED-3-cleaved form of CED-9 prevents apoptosis in mammalian cells by localizing to the inner mitochondrial membrane.

During the development of the nematode Caenorhabditis elegans (C. elegans), 131 of the 1090 somatic cells which are generated undergo programmed cell death (PCD) (SULSTON and HORVITZ, 1977; KIMBLE and HIRSH, 1979; SULSTON et al., 1983). The regulation of this PCD is executed by cell death genes which are commonly known as ced-3, ced-4, and ced-9; the former of which are required, in order for PCD to occur (ELLIS and HORVITZ, 1986), whereas the latter is required to protect cells that normally survive the process of PCD (HENGARTNER et al., 1992).

CED-4 has been shown to directly interact with both CED-3 and CED-9; CED-9 prevents cell death by directly binding to CED-4, forming a complex and presumably giving rise to an inactive conformation (CHINNAIYAN et al., 1997a,b; WU et al., 1997a,b). CED-9 and CED-4 localize to the mitochondria in wild-type embryos of C. elegans, whereas CED-4 is dissociated from CED-9 and is localized in the perinuclear region when the cells in the embryos are induced to die (CHEN et al., 2000). Dissociated CED-4 is capable of activating caspase (SESHAGIRI and MILLER, 1997). To date, however, the subcellular localization of CED-9 in cells of C. elegans remains to be investigated.

A mammalian homologue of CED-4 has been shown to be Apaf-1, part of which has a domain which is homologous to CED-4 (ZOU et al., 1997; CHEN et al., 2000). Different from the interaction of CED-4 with CED-9 in C. elegans, Apaf-1 is not co-immunoprecipitated with anti-apoptotic Bcl-2 family members, although it is coprecipitated with procaspase-9 (MORISHI et al., 1999). In addition, on receiving a death-inducing stimulation, pro-caspase-9 in mammalian cells is recruited by Apaf-1 which has been activated via an interaction with cytochrome c released from mitochondria and dATP/ATP, and is thus activated. We have recently demonstrated that Bcl-2 is preferentially localized in the inner membrane of purified mitochondria from wild-type and bcl-2-transfected PC12 cells, and intact rat brains (GOTOW et al., 2000). In fact, in the absence of an interaction with Apaf-1 in mammalian cells, Bcl-2 acts as an anti-apoptotic factor by inhibiting the release of cytochrome c from mitochondria (ZAMZAMI et al., 1998).

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The overexpression of human \textit{bcl-2} partially inhibits ectopic cell death in the \textit{ced-9} (loss-of-function) mutant as well as normal PCD in \textit{C. elegans} (Vaux et al., 1992; Hengartner and Horvitz, 1994). However, the overexpression of \textit{ced-9} cannot protect apoptosis of mammalian cells (Hisahara et al., 1998). These data suggest that anti-apoptotic behavior of \textit{CED-9} may differ from that of \textit{Bcl-2} in mammalian cells. It has recently been shown that the \textit{CED-9} protein is a substrate for \textit{CED-3} which is able to cleave the protein at two sites near its amino terminus, generating homologous proteins of \textit{Bcl-2} (Xue and Horvitz, 1997). Therefore, in the present study, we report here an examination of the effects of \textit{CED-3}-cleaved \textit{CED-9} on the viability of mammalian cells in order to better understand the mechanisms of why \textit{CED-9} is not able to protect mammalian cells from apoptosis, as induced by the appropriate death stimuli. For these experiments, PC12 cells and HeLa cells were prepared, both of which overexpressed the full-length and amino terminal truncated (68-280) forms of \textit{CED-9} (full-length \textit{CED-9} and \textit{CED-9}-), respectively. \textit{CED-9} effectively rescued apoptosis of these mammalian cells, but full-length \textit{CED-9} did not. Moreover, when these cells were rescued, the molecule behaved in a manner similar to \textit{Bcl-2} and was preferentially localized to the inner mitochondrial membrane.

**MATERIALS AND METHODS**

**Cell culture**

PC12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing a high level of glucose (4.5 g/l), while HeLa cells were cultured in RPMI1640. Both media were supplemented with 10% heat-inactivated horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). For the preparation of transfected cells, cDNAs of human \textit{bcl-2}, \textit{ced-9}, and truncated \textit{ced-9}, which encodes for the 68-280 amino acid sequence of \textit{CED-9} were inserted into pcDNA3 plasmid (Invitrogen). These constructs were transfected into PC12 and HeLa cells using the calcium phosphate precipitation method (Chen and Oka- yama, 1987), respectively and were confirmed to stably express their mRNAs and proteins. For the detection of proteins, \textit{ced-9} and the truncated \textit{ced-9} cDNAs used for the transfection were linked with the FLAG epitope tag sequence at sites corresponding to the NH2-terminus of full-length and the truncated forms of \textit{CED-9}.

**Antibodies**

Polyclonal and monoclonal antibodies against human and rat Bcl-2 were used, as previously reported (Gotow et al., 2000). Polyclonal antibodies against subunit β of mitochondrial ATP synthase (Ezaki et al., 1996) and Tom20 (Iwahashi et al., 1997) were used for marker proteins of the inner and outer mitochondrial membranes. A commercially available monoclonal antibody against the FLAG epitope (M2) (Sigma) was used for the detection of FLAG-tagged full-length and truncated \textit{CED-9}.

**Cell death assay**

Wild-type, and full-length \textit{ced-9}, \textit{CED-9}- or \textit{bcl-2}-transfected PC12 cells were cultured under serum deprivation. At 24 h after the beginning of cultures the cells were collected and their lactate dehydrogenase activities were spectrophotometrically measured. As controls, these cells were also cultured in the absence of serum but in the presence of 100 ng/ml NGF. The survival rates of these cells were determined by estimating those of cells cultured in the presence of NGF as 100%. In the case of wild-type, and full-length \textit{ced-9}, \textit{CED-9}- or \textit{bcl-2}-transfected HeLa cells, they were cultured in the presence of 10 ng/ml recombinant murine TNF-α (amino terminal truncated form, R & D System) and 5 μg/ml cycloheximide (Sigma) and the MTT tetrazolium assay was measured 12 h after the beginning of cultures, using an MTT assay kit (Promega). These HeLa cells which were cultured in the presence of TNF-α alone were used as controls and their survival rates were estimated as 100%. These experiments were carried out in triplicate, and the data were statistically analyzed by the student’s t-test.

**Immunohisto/cytchemistry**

**Confocal laser scanning microscopy**

Transfected PC12 and HeLa cells, plated on Chamber Slides (Nunc), were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1h at room temperature (RT) (Gotow et al., 2000). Transfected PC12 cells cultured under serum deprivation for 24 h and transfected HeLa cells cultured in the presence of TNF-α and cycloheximide for 12 h were also fixed with the same fixative for 1 h at RT. After washing with PBS they were incubated overnight at 4°C with both monoclonal anti-FLAG-tag and polyclonal anti-subunit β antibodies diluted in PBS containing 0.05% Tween 20, and then with anti-mouse IgG coupled with FITC and anti-rabbit IgG with Texas Red (Biomedia) for colocalization studies.

To examine the correlation of DNA fragmentation and the expression of full-length CED-9 or \textit{CED-9} in transfected PC12 and HeLa cells after appropriate
death stimuli, double staining of nick end labeling (Texas Red labeling) and FLAG-tag (FITC labeling) were performed, as previously reported (Isahara et al., 1999). To detect nuclear DNA fragmentation, the TUNEL reaction was applied to the fixed cells according to the modified method (Gavriel et al., 1992; Nitatori et al., 1995). Briefly, cells were incubated with 100 U/ml TdT and 10 nmol/ml biotinylated 16-2'-dUTP (Boehringer-Mannheim-Yamanouchi) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 μg/ml gelatin) in a humid atmosphere at 37°C for 60 min. Further incubation with Texas red-conjugated avidin (Nichirei) was carried out for 30 min at RT.

**Immunoelectron microscopy**

Immunocytochemical analysis using electron microscopy was carried out with cryo-thin section immunogold labeling (Gotow et al., 1996, 2000), originally designed by Tokuyasu (1980). Transfected PC12 and HeLa cells plated on the dishes, were fixed first with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at RT and then with 4% paraformaldehyde alone in the same buffer for 30 min. The fixed cells were washed with the buffer, scraped and centrifuged at 250 g for 5 min. Pelleted cells were suspended with 10% gelatin in 0.1 M phosphate buffer and again centrifuged at 1,200 g for 5 min. Gelatin-embedded cells were immersed in 2.3 M sucrose in 0.1 M phosphate buffer and frozen in liquid nitrogen.

Cryo-thin sections were cut with an ultramicrotome (Reichert-Nissei ULTRACUT S, Nissei Sangyo Co. Ltd.). Sections on grids were treated with 1% BSA in PBS, incubated with anti-FLAG overnight and with the secondary antibody coupled to 5 nm colloidal gold particles (Amersham) for 1 h. For double labeling, some grids were also incubated with both monoclonal anti-FLAG and polyclonal anti-subunit β, and then with anti-mouse IgG coupled with 5 nm gold particles and anti-rabbit IgG coupled with 15 nm gold particles (Amersham). The grids were then treated with 2% glutaraldehyde, 1% OsO4, and 1% uranyl acetate. They were then dehydrated, embedded in LR white and examined using a Hitachi H-7100 electron microscope.

**Western blotting**

PC12 and HeLa cells which express CED-9 and ΔCED-9 were rinsed with PBS after removal of the medium, scraped from the dishes and centrifuged at 250 g for 5 min at 4°C, respectively. The pellets were lysed with a lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100 including the proteinase inhibitor cocktail (Boehringer Mannheim). After being centrifuged twice at 10,500 g for 10 min at 4°C, the supernatants were measured for protein concentrations using the BCA protein assay system (Pierce) and immunoblotting was performed. For the detection of full-length CED-9 and ΔCED-9, each sample was separated by 12.5% SDS-PAGE. Electrophoretic transfer of proteins from polyacrylamide gels to a PVDF membrane (Immobilon-P; Millipore) was performed according to the method of Towbin et al. (1979). The sheets were soaked in PBS containing 5% bovine serum albumin (Sigma), in order to block non-specific binding, and then incubated with anti-FLAG-tag. Immunodetection was carried out with a chemiluminescent ECL kit (Amersham) according to the manufacturer’s recommended protocol. Protein levels were determined by scanning densitometry.

**Subcellular fractionation**

PC12 and HeLa cells which express CED-9 and ΔCED-9 were rinsed with PBS after removal of the medium, scraped from dishes and centrifuged at 250 g for 5 min at 4°C, respectively. Pelleted cells were homogenized in 0.25 M sucrose by passing 6 times through a 27 gauge needle at 4°C, and then centrifuged at 1,200 g for 5 min at 4°C. The supernatant was used as the postnuclear supernatant (PNS). PNS was loaded on a cushion of 15% (for PC12 cells) or 10% (for HeLa cells) Percoll in 0.25 M sucrose and, centrifuged at 50,000 g for 30 min, and fractionated into 12 fractions from bottom to top. These fractions were then incubated with lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100 including the proteinase inhibitor cocktail (Boehringer Mannheim) and subjected to SDS-PAGE and immunoblotting.

**Purification of mitochondria**

Mitochondria were isolated from the PNS of PC12 and HeLa cells which express full-length CED-9 or ΔCED-9 by centrifugation at 50,000 g for 1 h, using a hybrid Percoll-metrizamide discontinuous density gradient (Storrie and Madden, 1990). After removal of metrizamide by centrifugation at 12,500 g for 15 min, the mitochondrial fraction was suspended in 0.1 M Tris·HCl buffer (pH 7.4), containing 1 mM EDTA and 0.32 M sucrose for trypsin digestion. Purified mitochondria were treated with trypsin at concentrations of 0, 5, 10, 25, and 50 μg/ml in the reaction buffer for 20 min at 4°C. The samples were centrifuged at 15,000 g for 20 min after stopping the reaction, and pellets and supernatants were applied to SDS-PAGE and immunoblotting.
Fig. 1. Legend on the opposite page.
RESULTS

Viability of PC12 cells which express full-length or amino terminal (1–67)-truncated CED-9

To examine the anti-apoptotic activity of the full-length and CED-3-cleaved forms of CED-9 in mammalian cells, PC12 cells were prepared, which express full-length CED-9 and ΔCED-9, respectively, both of which were FLAG-tagged at their amino terminal ends (Fig. 1A). We first confirmed the expression of full-length CED-9 and ΔCED-9 in the PC12 cells by Western blotting and confocal laser scanning microscopy. Protein bands which were immunoreactive for the FLAG-tag, indicating full-length CED-9 and ΔCED-9, appeared at the molecular weights of 31 kDa and 26 kDa in extracts of both full-length ced-9- and Δced-9-transfected cells, respectively (Fig. 1B). Double immunostaining of subunit β of mitochondrial F$_{1}$F$_{0}$ATPase (subunit β) and the full-length or truncated forms of CED-9 demonstrated that immunoreactivity for the FLAG-tag showing the full-length or truncated forms of CED-9 was diffuse or, to some extent, granular in the cytoplasm of PC12 cells, while granular staining of subunit β was co-localized well with immunoreactivity showing full-length CED-9 or ΔCED-9 (Fig. 1C).

The viability of PC12 cells which express full-length CED-9 as well as that of the wild-type PC12 cells were determined 24 h after the beginning of serum-free culturing by measuring the lactate dehydrogenase activity in the surviving cells. The survival rates of these cells were similarly low, compared to those of cells which were cultured in the presence of NGF (Fig. 2A). On the contrary, the survival rate of PC12 cells which express ΔCED-9 was significantly increased relative to the cells expressing full-length CED-9 and the wild-type cells, when examined 24 h after the beginning of serum-deprived culturing (Fig. 2A). To examine the issue of whether ΔCED-9 also

Fig. 1. Expression of full-length (Full) CED-9 and ΔCED-9 in PC12 and HeLa cells. A. Schematic drawings of full-length CED-9 and ΔCED-9 molecules. Full-length CED-9 has a cleavage site of CED-3 at the position immediately after ESID (arrow). These two molecules possess Bcl-2 homologous domains (BH) and are tagged with FLAG at the NH$_{2}$-terminus. B. Immunoblot analysis. Protein bands which were immunoreactive for the FLAG-tag indicating full-length CED-9 or ΔCED-9 in extracts obtained from transfected PC12 and HeLa cells appear at 31 kDa for full-length CED-9 and 26 kDa for ΔCED-9. Proteins applied to SDS-PAGE were 30 µg in each lane. C. Double immunostaining images of subunit β of mitochondrial F$_{1}$F$_{0}$ATPase (Texas red, red) and full-length CED-9 or ΔCED-9 (FITC, green) in PC12 cells (left panel) and HeLa cells (right panel) by laser scanning microscopy. Immunoreactivity for a mitochondrial marker protein, subunit β is diffuse and, to some extent, granular in the cytoplasm of PC12 cells, while it is diffuse or string-like in the cytoplasm of HeLa cells. Positive staining for the FLAG-tag (FLAG) indicating full-length CED-9 and ΔCED-9 shows similar localization patterns in PC12 cells and HeLa cells, respectively. Overlay images (yellow) of subunit β and full-length CED-9 or ΔCED-9 clear-cut co-localization of these proteins in both cells. PC12 cells: ×450, HeLa cells: ×350

Fig. 2. The viability of wild-type (wild) PC12 cells (A) and HeLa cells (B), and cells expressing full-length (full) CED-9, ΔCED-9 or Bcl-2. The survival rates of PC12 and HeLa cells expressing ΔCED-9 are significantly higher than wild-type cells and those expressing full-length CED-9, respectively, whereas those of cells expressing ΔCED-9 are lower than those of Bcl-2-expressing cells. The transfected PC12 cells were cultured in the absence of serum for 24 h and the LDH activity within the living cells were measured. These cells cultured under the presence of NGF were used as controls and their LDH activity was estimated as 100%. The transfected HeLa cells were cultured in the presence of 10 ng/ml recombinant murine TNF-α and 5 µg/ml cycloheximide and the MTT tetrazolium assay was measured 12 h after the beginning of cultures. Those HeLa cells cultured in the presence of TNF-α alone were used as controls and their survival rates were estimated as 100%. These experiments were carried out in triplicate. Bars indicate ± standard deviation.
acts as an anti-apoptotic factor in cells other than PC12 cells. HeLa cells which express ΔCED-9 or full-length CED-9 were prepared. The expression of full-length CED-9 and ΔCED-9 in transfected HeLa cells was confirmed by both Western blotting and confocal laser scanning microscopy (Fig. 1). In particular, a diffuse or string-like staining of subunit β was largely co-localized with that of the FLAG-tag, indicating the presence of full-length CED-9 or ΔCED-9. The survival rate of HeLa cells which express ΔCED-9 was much higher than those of the cells expressing full-length CED-9 and wild-type cells, when these cells were cultured in the presence of 10 ng/ml TNF-α and 5 μg/ml cycloheximide for 12 h, as evidenced by a measurement of viability of the cells were measured by the MTT assay (Fig. 2B). In both the transfected PC12 and HeLa cells, however, the viability of the cells which express ΔCED-9 was much lower than that of the cells expressing Bcl-2 (Fig. 2). These results indicate that the full-length form of CED-9 cannot rescue apoptosis of mammalian cells, but the CED-3-cleaved form (68-280) of CED-9 acts as an anti-apoptotic factor in the cells.

To confirm survival and apoptotic cells, double staining of TUNEL and full-length or truncated CED-9 was performed. As shown in Figure 3, dual-positive PC12 and HeLa cells for TUNEL and full-length CED-9 were clearly present in larger numbers than those for TUNEL and ΔCED-9 after appropriate death stimuli, respectively.

Subcellular distribution of full-length CED-9 and ΔCED-9 expressed in PC12 and HeLa cells

As shown in Figure 1C, diffuse and/or granular immunolocalization of full-length CED-9 and ΔCED-9 was detected in the cytoplasm of PC12 and HeLa cells by confocal laser scanning microscopy. Moreover, the immunoreactivity for Flag-tag in both cases showed no evidence for any perinuclear staining patterns (Fig. 1C). To further examine the subcellular localization of full-length CED-9 and ΔCED-9 in PC12 and HeLa cells, immunocytochemical analyses using the cryo-thin section immunogold method were performed. Gold particles showing ΔCED-9 were largely co-localized with those, indicating the presence of subunit β in the inner mitochondrial membrane of PC12 and HeLa cells but rarely in the outer membrane (Fig. 4). Labeling of ΔCED-9 was also associated with the smooth endoplasmic reticulum (sER) in these cells but not with the nuclear envelope (data not shown). The full-length form of CED-9 was immunocytochemically demonstrated to be mainly in membranes of sER in both PC12 and HeLa cells, while gold labeling of full-length CED-9 was much less in number but detectable in the outer mitochondrial membrane in PC12 cells (Fig. 4). In some cases, however, labeling of full-length CED-9 and ΔCED-9 was evident in the rough ER, especially in HeLa cells (data not shown). Immunohisto/cytochemical studies using PC12 and HeLa cells suggest that ΔCED-9 is largely associated with the inner membrane of mitochondria, whereas full-length CED-9 is localized in the outer membrane to some extent, and largely in the sER membrane.

To confirm the immunocytochemical results, subcellular fractionations of postnuclear supernatants (PNS) from PC12 and HeLa cells expressing full-length CED-9 or ΔCED-9 were performed by the 15% Percoll gradient for PC12 cells and 10% for HeLa cells, respectively. As shown in Figure 5A, full-length CED-9 in PC12 cells were bimodally distributed in the heavy membrane fractions, which correspond to the mitochondrial fractions and in light membrane fractions, which correspond to the ER/vesicular fractions. ΔCED-9 in the cells was localized mostly in the heavier fractions (Fig. 5A). In HeLa cells, ΔCED-9 showed a bimodal distribution pattern with the heavy mitochondrial and the light membrane fractions, but full-length CED-9 was detected largely in the light membrane fractions (Fig. 5B).
**ΔCED-9 in purified mitochondria is resistant to digestion by trypsin, but full-length CED-9 is not**

The association of ΔCED-9 with the inner mitochondrial membrane was further examined by digesting purified mitochondria with trypsin. As marker proteins of the outer and inner mitochondrial membranes, a mitochondrial import receptor protein, Tom20 (IWASHI et al., 1997) and subunit β of mitochondrial F$_{1}$F$_{0}$ATPase, were used, respectively. Using this trypsin digestion method, we previously demonstrated that Bcl-2 in purified mitochondria from rat brains and PC12 cells is localized to the inner membrane (GOTOW et al., 2000). Therefore, we also examined the resistance of endogenous Bcl-2 to trypsin digestion as a control (data not shown). When purified mitochondria from PC12 cells which express ΔCED-9 were treated with increasing concentrations of trypsin, ΔCED-9 and subunit β were resistant to the digestion up to concentrations of 50 μg/ml, whereas Tom20 was almost completely digested at a concentration of 10 μg/ml (Fig. 6A, B). The proportions of the amounts of non-digested protein after treatment with 50 μg/ml trypsin were 87.0% in ΔCED-9 and 100% in subunit β (Fig. 6B). In the case of purified mitochondria from the cells which express full-length CED-9, subunit β also showed a similar tendency to that of ΔCED-9 against trypsin digestion, while Tom20 was also digested by a concentration of 10 μg/ml trypsin. Full-length CED-9, however, was largely digested at a concentration of 10 μg/ml trypsin and nearly disappeared when a concentration of 25 μg/ml trypsin was used (Fig. 6C, D). The proportions of the amounts of non-digested full-length CED-9.
after treatment with 10 and 25 μg/ml trypsin were 29.0% and 11.3%, respectively (Fig. 6D). In HeLa cells, full-length CED-9 could not be detected in purified mitochondria, although ΔCED-9 was also resistant to trypsin digestion up to a concentration of 50 μg/ml (data not shown). The results again suggest that ΔCED-9 is associated with the inner mitochondrial membrane.

**DISCUSSION**

The present study demonstrated that full-length CED-9 failed to prevent apoptosis of PC12 and HeLa cells when overexpressed in these mammalian cells, whereas the CED-3-cleaved form of CED-9 (ΔCED-9) significantly suppressed apoptosis of these cells. Moreover, ΔCED-9 is localized to the mitochondrial inner and smooth ER-vesicular membranes, while full-length CED-9 was detected mainly in smooth ER-vesicular membranes in PC12 and HeLa cells and in the outer mitochondrial membrane in PC12 cells.

A gain-of-function mutation in the gene ced-9 suppresses PCD in *C. elegans* embryos, whereas it cannot survive mammalian cells from apoptosis (HISAHARA et al., 1998). This latter phenomenon is consistent in the cases of PC12 and HeLa cells as demonstrated in the present study. The question raised in the present study was why full-length CED-9 is not able to block apoptosis of mammalian cells. It is well known that Bcl-2, a mammalian counterpart of CED-9, is able to suppress the apoptosis of both PC12 and HeLa cells, suggesting the possibility that these two molecules differ from each other in their functional sites in mammalian cells.

A confocal laser scanning microscopic study has clearly demonstrated that both CED-9 and CED-4 are co-localized with Mitotracker in wild-type *C. elegans* embryos, respectively (CHEN et al., 2000). When EGL-1, a proapoptotic regulator, is expressed in the embryos, the localization of CED-9 remains in the mitochondria, but that of CED-4 is translocated in the perinuclear region (CHEN et al., 2000), suggesting that CED-9 is localized to the outer mitochondrial membrane by its transmembrane domain. The present electron microscopic and biochemical studies confirm this localization pattern of full-length CED-9 in PC12 cells, although the localization was mainly detected in ER-vesicular membranes in HeLa cells. These results indicate that even if full-length CED-9 is localized to the outer mitochondrial membrane, it cannot prevent mammalian cells from apoptosis induced by

![Fig. 5. Subcellular fractionation of PNS from PC12 and HeLa cells expressing full-length (Full) CED-9 or ΔCED-9. The fractionation was performed on a Percoll gradient. Aliquots of 200 μl each were collected from the gradient and 10 μl aliquots of each were loaded, while they were subjected to SDS-PAGE and finally to immunoblotting by antibodies specific for FLAG-tag indicating full-length CED-9 and ΔCED-9, and subunit β of mitochondrial ATP synthase. A. PC12 cells. Full-length CED-9 in PC12 cells are distributed in the heavy membrane fractions corresponding to the mitochondrial fractions where subunit β is detected and weakly in the light membrane fractions, which correspond to the ER/vesicular fractions. ΔCED-9 in the cells is largely detected in the heavier fractions. B. HeLa cells. ΔCED-9 shows a bimodal distribution pattern with the heavy mitochondrial and the light membrane fractions, whereas full-length CED-9 is largely distributed in the light membrane fractions.](image-url)
appropriate death stimuli.

Different from the interaction between CED-9 and CED-4 or CED-3 in C. elegans embryos (Chinnaiyan et al., 1997a, b; Imler et al., 1997; Spector et al., 1997; Wu et al., 1997a, b), it has been shown that Apaf-1, a mammalian homologue of CED-4, is able to interact with procaspase-9, but not with the anti-apoptotic Bcl-2 family of proteins (Moriishi et al., 1999). This result suggests that Bcl-2 indirectly regulates the function of Apaf-1 (Moriishi et al., 1999). Moreover, anti-apoptotic Bcl-2 family members may suppress the release of apoptogenic factors from mitochondria (Green and Reed, 1998; Zamzami et al., 1998). However, the issue of precisely where prosurvival Bcl-2 family members function in the cells remains controversial, although a number of immunocytochemical, as well as biochemical studies, have shown that they function in membranes of the outer mitochondria, the rER and the nuclear envelope (Baffy et al., 1993; Krajewski et al., 1993; Akae et al., 1994; Lam et al., 1994; Ryan et al., 1994). However, using purified mitochondria from normal rat brains, and wild-type and bcl-2-transfected PC12 cells, we have been able to demonstrate that Bcl-2 is preferentially localized to the inner but not the outer

Fig. 6. Digestion of purified mitochondria from PC12 cells expressing full-length (Full) CED-9 or ΔCED-9 with trypsin. Immunoblotting of ΔCED-9, full-length CED-9, subunit β, and Tom20 in pellets (P) and supernatants (S) of purified mitochondria after treatment with trypsin at concentrations of 0, 5, 10, 25 and 50 μg/ml for 20 min on ice. A. PC12 cells expressing ΔCED-9. In samples, Tom20 is completely digested by 10 μg/ml trypsin, whereas ΔCED-9 and subunit β are resistant to trypsin even at concentrations of 50 μg/ml. B. Quantification of A. The blotted densities were measured with a Scanning Imager and proportions of non-digested amounts against the initial ones were determined. Proportions of non-digested protein amounts after treatment with 50 μg/ml trypsin were 87.8% in ΔCED-9 and 100% in subunit β. C. PC12 cells expressing full-length CED-9. Full-length CED-9 is largely digested at the concentration of 10 μg/ml trypsin and faintly detected at the concentration of 25 μg/ml, while the resistance of three other proteins to trypsin digestion shows patterns which are similar to those in A. D. Quantification of C. Proportions of the amounts of non-digested protein full-length CED-9 after treatment with 10 and 25 μg/ml trypsin were 29.8% and 11.3%, respectively. Vertical bars indicate ± standard deviation (n=3) in B and D.
mitochondrial and sER/vesicular membranes (Gotow et al., 2000). This result suggests that anti-apoptotic Bcl-2 family members might inhibit the apoptosis of mammalian cells by localizing to the inner mitochondrial membrane. To confirm this hypothesis, PC12 and HeLa cells were prepared, which overexpress the CED-3-cleaved form of CED-9 which contains a 68-280 amino acid sequence of CED-9 and is a homologous protein of Bcl-2, and examined their viability and intracellular localization.

As stated above, ΔCED-9 prevented PC12 and HeLa cells from apoptosis induced by death stimuli, respectively, although the anti-apoptotic activity of the protein in these cells was lower than that of Bcl-2. This lower activity of ΔCED-9 may be attributed to the dissimilarity of amino acid sequences between ΔCED-9 and Bcl-2 (the similarity of amino acid sequences between the two molecules is 34%). Since ΔCED-9 had anti-apoptotic activity, we further analyzed its subcellular localization. As expected, ΔCED-9 was preferentially localized to inner but not the outer mitochondrial and smooth ER/vesicular membranes in both types of mammalian cells, when examined by immunoelectron microscopy using the cryo-thin section immunogold method and by the subcellular fractionation analysis. According to previous immunocytochemical studies, which used the pre-embedding method, Bcl-2 has been shown to localize to ribosome-attached membranes such as the rER and nuclear envelope in addition to the outer mitochondrial membrane (Krajewski et al., 1993; Akao et al., 1994). However, as the localization of Bcl-2 has been demonstrated in our previous study (Gotow et al., 2000), ΔCED-9 also rarely localized to the nuclear envelope and the rER.

To confirm the present data obtained by immunocytochemical and subcellular fractionation analyses, a digestion study was performed with trypsin, which is considered to be the most important experiment in determining which sides of mitochondrial membranes, the inner or outer, ΔCED-9 is localized. According to our previous study (Gotow et al., 2000), we carefully isolated mitochondria from PC12 and HeLa cells which express ΔCED-9, respectively, and digested them with increasing concentrations of trypsin. Full-length CED-9 as well as a mitochondrial import receptor protein, Tom20 were resistant to trypsin digestion up to concentrations of 10 µg/ml, whereas not only endogenous Bcl-2 but also ΔCED-9 and subunit β were resistant to treatment with 50 µg/ml trypsin. These lines of evidence thus argue strongly for the preferential association of ΔCED-9 with the inner membrane compartment, even in the case of purified mitochondria. This trypsin digestion study also suggests that full-length CED-9 is localized to the outer membrane in PC12 cells.

The cleavage of CED-9 by CED-3 has been demonstrated in an in vitro study by Xue and Horvitz (1997) although the issue of whether this cleavage occurs in vivo remains largely unknown. The regulation of CED-4 by CED-9 has been shown to occur at the cytoplasmic face of the outer mitochondrial membrane in embryonic cells of C. elegans (Chen et al., 2000). This anti-apoptotic regulation by CED-9 was not functional in mammalian cells, as shown in the present study. On the contrary, ΔCED-9 prevented apoptosis in mammalian cells by localizing to the inner mitochondrial membrane. In addition to our previous study concerning the localization of Bcl-2, the protein has been shown to be localized predominantly to the inner mitochondrial membrane by the cryo-thin section immunogold method (Motoyama et al., 1998), while Bcl-XL has been demonstrated to bind specifically to cytochrome c and has been suggested to be localized within mitochondria (Kharbanda et al., 1997). In fact, Bcl-2 and Bcl-XL have been shown to repress the processes of both apoptosis and some forms of chemical hypoxia-induced necrosis through blocking the mitochondrial membrane potential (Shimizu et al., 1996). Collectively, the present data showing that, as opposed to full-length CED-9, ΔCED-9 blocked apoptosis of mammalian cells are consistent with our hypothesis that Bcl-2 is preferentially localized to the inner mitochondrial membrane. Moreover, considering the fact that the release of cytochrome c from the inner mitochondrial membrane is one major consequence of the loss of mitochondrial potential (Zamzami et al., 1996), it seems likely that the selective localization of Bcl-2 to the inner mitochondrial membrane plays a key role in the maintenance of physiological function in mitochondria, as well as the prevention of apoptosis.

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