Caspase-9 processing by caspase-3 via a feedback amplification loop *in vivo*

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Abstract

In contrast to the autoprocessing of caspase-9, little is known about the biological significance of caspase-9 processing by caspase-3 via a feedback loop *in vivo*. We prepared antisera against mouse caspase-9 cleavage sites so that only the activated form of mouse caspase-9 was recognized. Using these antisera and caspase-9- and caspase-3-deficient mouse embryonic fibroblasts, we demonstrated that mouse caspase-9 is initially autoprocessed at D³⁵³ and D³⁶⁸ at low levels during staurosporine-induced apoptosis, whereupon the D³⁶⁸ and D¹⁶⁸ sites are preferentially processed over D³⁵³ by activated caspase-3 as part of a feedback amplification loop. Ac-DEVD-MCA (caspase-3-like) and Ac-LEHD-MCA (caspase-9-like) cleavage activities clearly showed that caspase-9 autoprocessing was necessary for the activation of caspase-3, whereas full activation of caspase-3 and caspase-9 was achieved only through the feedback amplification loop. This feedback amplification loop also played a predominant role during programmed cell death of dorsal root ganglia neurons at mouse embryonic day 11.5. *Cell Death and Differentiation* (2001) 8, 335–344.

Keywords: apoptosis; caspase-9; caspase-3; Ced-3; staurosporine

Abbreviations: MEF; mouse embryonic fibroblasts

Introduction

Members of the caspase family, homologues of Ced-3 in *Caenorhabditis elegans* (*C. elegans*), are essential components of the mammalian cell death pathway.¹ Caspases are synthesized as inactive precursors and upon stimulation by apoptotic signals, are activated in a sequential cascade in which upstream (initiator) caspases process downstream (effector) caspases.² Caspase-9 is an initiator caspase that is autoprocessed into its active form by oligomerization via an N-terminal caspase recruitment domain (CARD),³ while caspase-3 is the downstream effector caspase.

Many apoptotic stimuli, including staurosporine (ST),⁴ cause cell death by inducing cytochrome c release, which together with Apaf-1 and ATP/dATP, facilitate caspase-9 processing and initiate the caspase cascade.⁵ In the presence of dATP and cytochrome c,^{6,7} the CARD domains of caspase-9 and Apaf-1 interact to produce an apoptosome complex,^{8,9} defined as an Apaf-1-containing complex that catalyzes the activation of caspases. Procaspase-9 is then autoprocessed into its active form and in turn, activates caspase-3. Interaction between caspase-9 and Apaf-1 is an essential component of both p53-regulated apoptosis¹⁰ and programmed cell death during brain development.^{11–14}

Mouse caspase-9 (mcasepase-9) is thought to have two putative cleavage sites, D³⁵³ (SEPD) and D³⁶⁸ (DQLD) that are similar to the human caspase-9 (hcasepase-9) cleavage sites D³¹⁵ (PEPD) and D³³⁰ (DQLD), respectively.¹⁵ Human caspase-9 is autoprocessed at D³¹⁵ and processed at D³³⁰ by caspase-3 *in vitro*.^{16,17} Recently, it has been shown using a cell-free system that activated caspase-3 acts on caspase-9 processing in a feedback amplification loop that results in complete activation of caspase-9.¹⁸ However, little is known of the biological significance of the caspase-9/-3 feedback amplification loop in apoptosis and programmed cell death during development.

Antibodies directed against the cleavage site of mcasepase-3 (anti-p20/17, here referred to as anti-m3D175) have been generated¹⁹ and are able to detect the processing of mcasepase-3 at D¹⁷⁵ during both programmed cell death associated with the development of the nervous system^{20,21} and TNF- or retinoic acid-induced apoptosis.^{22,23} In the present study, we attempted to clarify *in vivo* mcasepase-9 processing by the feedback amplification loop.

We used antisera against putative mcasepase-9 cleavage sites, as well as caspase-9 (–/–) and caspase-3 (–/–) mouse embryonic fibroblasts (MEF) and embryos, and showed that mcasepase-9 processing by the feedback amplification loop was necessary for constitutive and full activation of both mcasepase-9 and -3 during ST-induced apoptosis and programmed cell death of dorsal root ganglia (DRG) neurons in mouse embryos.

Results

Specificities of anti-m9D353 and anti-m9D368

We prepared antisera against peptides derived from the mcasepase-9D³⁵³ and D³⁶⁸ cleavage sites. Anti-m9D353 reacted specifically with the 39 kDa FLAG-mcasepase-9D353 fusion protein (p39), composed of FLAG (1 kDa) and mcasepase-9D353 (38 kDa). Anti-m9D368 reacted specifically with FLAG-mcasepase-9D368 (p40), a 40 kDa fusion protein composed of FLAG (1 kDa) and mcasepase-9D368 (39 kDa). Neither antisera reacted with the processing

fragments of other caspases, including caspase-2, -3, -6, -7, and -8 (Figure 1).

When FLAG-mcaspase-9 was transfected into COS cells, FLAG-mcaspase-9 (p50) appeared after 12 h, at which point the processing fragments (p39 to 40) were only faintly observable, but increased in a time-dependent manner. While neither anti-m9D353 nor anti-m9D368 reacted with p50, anti-m9D353 and anti-m9D368 reacted with p39 and p40 fragments, respectively, at 12 h and reactivities increased at 18 to 24 h. Processing fragments with molecular weights of 20–21 kDa (provisionally referred to as p20–21), which reacted with anti-m9D368 but not with anti-FLAG, were observed 24 h after transfection (Figure 2A).

Anti-m9D353 and anti-m9D368 were also used for immunochemical detection of EGFP-mcaspase-9 D³⁵³ and D³⁶⁸ processing fragments (Figure 2B). Cells expressing EGFP-mcaspase-9D353 and EGFP-mcaspase-9D368 were

used as positive controls for anti-m9D353 and -m9D368 immunostaining, and showed no apoptotic features 24 h after transfection, but exhibited strong immunoreactivity against anti-m9D353 and -m9D368, respectively. While most cells expressing EGFP-mcaspase-9 were non-apoptotic at 12 h, some cells showed apoptotic features with cell shrinkage at 24 h. Whereas anti-m9D353 and anti-m9D368 staining was negative in most EGFP-mcaspase-9 expressing cells 12 h after transfection, at 24 h positive staining was observed in most apoptotic cells expressing EGFP-mcaspase-9.

Detection of the processing of mcaspase-9 in ST-treated wildtype (WT), caspase-3 (–/–) and caspase-9 (–/–) MEF cells

ST is known to induce caspase-9 activation via cytochrome c release from mitochondria.^{4,5} While high Ac-LEHD-MCA (caspase-9-like) and Ac-DEVD-MCA (caspase-3-like) clea-

Figure 1 Immunoblot analyses of anti-m9D353 and anti-m9D368 antibody specificities. FLAG-mcaspase-9D353, -9D368 and the active processing fragments of other caspases were transfected into COS cells, and the reactivities to anti-m9D353 and anti-m9D368 examined by immunoblot analysis using anti-FLAG, anti-m9D353, anti-m9D368, and anti-m3D175. Lane 1; FLAG-rcaspase-2D394, lane 2; FLAG-mcaspase-3D175, lane 3; FLAG-mcaspase-6D162, lane 4; FLAG-mcaspase-7D198, lane 5; FLAG-mcaspase-8D387(-DED), lane 6; FLAG-mcaspase-9D353, lane 7; FLAG-mcaspase-9D368

vage activities were detected in ST-treated WTMEF cells, Ac-DEVD-MCA cleavage activity was not detected in *caspase-9* (−/−) and *caspase-3* (−/−) MEF cells (Figure 3A). Interestingly, Ac-LEHD-MCA cleavage activity was markedly inhibited in *caspase-3* (−/−) MEF cells.

To determine whether the p20–21 fragments that reacted with anti-m9D368 were processing fragments of mcaspase-9, we analyzed mcaspase-9 processing in ST-

treated WT, *caspase-9* (−/−) and *caspase-3* (−/−) MEF cells by immunoblot analysis using anti-m9D353 and anti-m9D368 (Figure 3B). Anti-m3D175 reacted with p17, a mcaspase-3 processing fragment, in ST-treated WT MEF cells (lane 2), but not in *caspase-9* (−/−) and *caspase-3* (−/−) MEF cells (lanes 4 and 6). Anti-m9D353 and anti-m9D368 did not react with p50 in untreated or ST-treated WT MEF cells, but did react with p38 and p39 in ST-treated

Figure 2 Detection of mcaspase-9 processing fragments by anti-m9D353 and anti-m9D368. **(A)** Immunoblot analysis of mcaspase-9 processing fragments. After FLAG-mcaspase-9 was transfected into COS cells, the expression and processing of FLAG-mcaspase-9 was examined in a time-dependent manner by immunoblot analysis using anti-FLAG, anti-m9D353, and anti-m9D368. **(B)** Immunocytochemical analysis of EGFP-mcaspase-9 processing. After EGFP-mcaspase-9, -m9D353, and -m9D368 were transfected into COS cells, the processing of mcaspase-9 was examined at 12 and 24 h by double staining of EGFP (green) and anti-m9D353 or anti-m9D368 (red; Texas-Red). Bars indicate 10 μm

WT MEF cells (lane 2). p38 and p39 were not detected in ST-treated *caspase-9* ($-/-$) MEF cells (lane 4), but were present in ST-treated *caspase-3* ($-/-$) MEF cells (lane 6). In addition, anti-m9D368 showed stronger reactivity to p20–21 fragments than p39 in ST-treated WT MEF cells (lane 2). Anti-m9D353 also showed weak reactivity to p20–21 (lane 2). However, p20–21 fragments reacting with anti-m9D353 or anti-m9D368 were not detected in either ST-treated *caspase-9* ($-/-$) MEF cells (lane 4) or ST-treated *caspase-3* ($-/-$) MEF cells (lane 6).

To confirm that the p20–21 fragments were N-terminal processing fragments of mcaspase-9, we also prepared antiserum, anti-m9D168, against the putative processing site at D¹⁶⁸ (Figure 4A). Anti-m9D168 specifically recognized FLAG-mcaspase-9D168 (p19) (lane 4), a 19 kDa fusion protein composed of FLAG (1 kDa) and mcaspase-

9D168 (18 kDa), but not FLAG-mcaspase-9 (p50), -mcaspase-9D353 (p39) or -mcaspase-9D368 (p40). As shown in Figure 4B, anti-m9D168 reacted with an 18 kDa processing fragment (p18) in ST-treated WT MEF cells (lane 2) but not in ST-treated *caspase-9* ($-/-$) and *caspase-3* ($-/-$) MEF cells (lanes 4 and 6). Thus, mcaspase-9 appears to be preferentially processed by activated caspase-3 at D³⁶⁸ and D¹⁶⁸ via a feedback loop.

Immunohistochemical detection of feedback activation of mcaspase-9 by mcaspase-3 in ST-mediated apoptosis and programmed cell death of DRG neurons in mouse embryos

Immunoblot analysis suggested that anti-m9D168 was more useful than anti-m9D368 to detect mcaspase-9 processing

Figure 3 Processing of mcaspase-9 in ST-treated cells. (A) Ac-LEHD-MCA (caspase-9-like activity) and Ac-DEVD-MCA (caspase-3-like activity) cleavage activity. After WT, *caspase-9* ($-/-$) and *caspase-3* ($-/-$) MEF cells were treated with 1 μ MST for 24 h, Ac-LEHD-MCA (upper panel) and Ac-DEVD-MCA (lower panel) cleavage activities were measured. The values are the average of three measurements. Bars indicate standard deviation. (B) Immunoblot analysis of the processing fragments of mcaspase-9. Processing fragments of mcaspase-9 and mcaspase-3 were examined by immunoblot analysis using anti-m9D353, anti-m9D368, and anti-m3D175. Anti-PKC ϵ was used as a control. Lanes 1, 3 and 5; untreated MEF cells, lanes 2, 4 and 6; ST-treated MEF cells. Lanes 1 and 2; WT MEF cells, lanes 3 and 4; *caspase-9* ($-/-$) MEF cells, lanes 5 and 6; *caspase-3* ($-/-$) MEF cells. *Indicates an unidentified processing fragment reacting with anti-m9D368 in ST-treated *caspase-3* ($-/-$) MEF cells. This fragment was only observed in ST-treated *caspase-3* ($-/-$) MEF cells but not in ST-treated WT MEF cells, suggesting that this is a fragment of mcaspase-9 processed by other caspases compensating for caspase-3 in *caspase-3* ($-/-$) MEF cells

fragments in the feedback loop, as unlike anti-m9D368, anti-m9D168 did not react with mcaspase-9 autoprocessing fragments in ST-treated *caspase-3* ($-/-$) MEF cells. Unfortunately, since anti-m9D168 failed to show immunohistochemical reactivity (data not shown), we compared anti-m9D368 and anti-m3D175 reactivities by immunostaining ST-treated *WT*, *caspase-3* ($-/-$) and *caspase-9* ($-/-$) cells. Both anti-m9D368 and anti-m3D175 reactivities were observed in ST-treated *WT* MEF cells that showed apoptotic features (Figure 5A, b and d). In contrast, anti-m9D368 and anti-m3D175 reactivities were not detected in ST-treated *caspase-9* ($-/-$) MEF cells with morphological alterations (Figure 5A, j and l). Under the same conditions used to detect anti-m9D368 reactivity in ST-treated *WT* MEF cells, anti-m9D368 reactivity was only weakly and rarely detected in ST-treated *caspase-3* ($-/-$) MEF cells (Figure 5A, f and h). Anti-m9D368 reactivity in ST-treated *caspase-3* ($-/-$) MEF cells could only be detected after long incubation times with anti-m9D368 (Figure 5A, inset in f).

DRG neurons undergo programmed cell death during embryogenesis, and anti-m3D175 reactivity has been detected in apoptotic DRG neurons during development.^{20,21} Anti-m9D368- and anti-m3D175-positive cells were detected in DRG of *WT* mouse embryos at E11.5 (Figure 5B, a and c), but undetectable in DRG of *caspase-3*

($-/-$) and *caspase-9* ($-/-$) mouse embryos (Figure 5B, e, g, i and k). Consistent with the reactivities of anti-m9D368 and anti-m3D175, TUNEL-positive cells were observed in DRG of *WT* mouse embryos (Figure 5B, a and c), but rarely observed in *caspase-3* ($-/-$) and *caspase-9* ($-/-$) mouse embryos (Figure 5B, e, g, i and k).

Discussion

Autoprocessing and processing of mcaspase-9

Mouse caspase-9 has two processing sites, D³⁵³ and D³⁶⁸, which are similar to the autoprocessing site (D³¹⁵) and the caspase-3 processing site (D³³⁰), respectively, of hcaspase-9.^{15–17} Mouse caspase-9 was processed at D³⁵³ and D³⁶⁸ into p38 and p39 in COS cells and ST-treated *WT* MEF cells (Figures 2A and 3B), and p38 and p39 were also detected in ST-treated *caspase-3* ($-/-$) MEF cells (Figure 3B), suggesting that mcaspase-9 is autoprocessed at both sites.

The main processing fragments of mcaspase-9 were small fragments (p20–21) reacting with anti-m9D368 in ST-treated *WT* MEF cells (Figure 3B). Human caspase-9 is processed at a site near the N-terminal end of the protein in addition to at D³¹⁵ and D³³⁰.²⁴ Similar to hcaspase-9,

Figure 4 Processing of N-terminal sites of mcaspase-9. **(A)** Preparation of antiserum against the putative D¹⁶⁸ cleavage site of mcaspase-9. FLAG-mcaspase-9 (lanes 1 and 3) and FLAG-m9D168 (lanes 2 and 4) were transfected into COS cells. After 18 h, the reactivity of anti-m9D168 was examined by immunoblot analysis using anti-FLAG (lanes 1 and 2) and anti-m9D168 (lanes 3 and 4). Anti-m9D168 reacted with FLAG-mcaspase-9D168 (p19) but not FLAG-mcaspase-9 (p50), -mcaspase-9D353 (p39) or -mcaspase-9D368 (p40). Closed arrow indicates the D¹⁶⁸ processing site. Open arrows indicate the putative mcaspase-9 processing sites, D¹⁷⁶ and D¹⁸⁸. **(B)** Immunoblot analysis using anti-m9D168 of mcaspase-9 processing in *WT*, *caspase-3* ($-/-$) and *caspase-9* ($-/-$) MEF cells treated with 1 μ M ST for 24 h. Lanes 1 and 2; *WT* MEF cells, lanes 3 and 4; *caspase-3* ($-/-$) MEF cells, lanes 5 and 6; *caspase-9* ($-/-$) MEF cells

mcaspase-9 also appears to be processed at sites near the N-terminus in addition to the D³⁵³ and D³⁶⁸ sites. In COS cells overexpressing FLAG-mcaspase-9, p20–21 did not appear concurrently with FLAG-mcaspase-9D368 (p40) but appeared later (Figure 2A). Anti-m9D368 reactive p20–21 fragments were much more prevalent than anti-m9D353 reactive fragments, suggesting that mcaspase-9D368 was

more susceptible to autoprocessing than mcaspase-9 or mcaspase-9D353. Large caspase fragments can be further autoprocessed to active forms, for example, processed caspase-3 (p20) is autoprocessed spontaneously into p3 and p17.²⁵ Thus, mcaspase-9D368 would give rise to the p20–21 fragments lacking the N-terminus of mcaspase-9 but having C-terminal D³⁶⁸.

Figure 5 Detection of caspase-9 activation by feedback amplification by immunostaining using anti-m9D368 and anti-m3D175. **(A)** Immunostaining using anti-m9D368 and anti-m3D175 of ST-treated WT, *caspase-3* (-/-) and *caspase-9* (-/-) MEF cells. After WT, *caspase-3* (-/-) and *caspase-9* (-/-) MEF cells were treated with 1 μ M ST for 24 h, anti-m9D368 and anti-m3D175 reactivities were examined by immunostaining (a to d); WT MEF cells, (e to h); *caspase-3* (-/-) MEF cells, (i to l); *caspase-9* (-/-) MEF cells. (a, c, e, g, i and k); untreated cells, (b, d, f, h, j and l); ST-treated cells. (a, b, e, f, i and j); anti-m9D368, (c, d, g, h, k and l); anti-m3D175. Insets show the pictures after long incubation times (for 72 h) with anti-m9D368. Arrows indicate anti-m9D368- or anti-m3D175-positive cells. Bars indicate 5 μ m. **(B)** Immunostaining using anti-m9D368 and anti-m3D175 of the DRG of WT, *caspase-3* (-/-) and *caspase-9* (-/-) mouse embryos. WT, *caspase-3* (-/-) and *caspase-9* (-/-) E11.5 mouse embryonic DRG were double-stained by TUNEL-labelling (red; Texas-Red) and anti-m9D368 or anti-m3D175 immunostaining (green; FITC). (a–d); WT, (e–h); *caspase-3* (-/-), (i–l); *caspase-9* (-/-). (a, e and i); anti-m9D368 and TUNEL, (c, g and k); anti-m3D175 and TUNEL, (b, d, f, h, j and l); phase contrast. Arrows (yellow) indicate anti-m9D368/TUNEL or anti-m3D175/TUNEL-positive cells. Bars indicate 100 μ m

However, anti-m9D353 and anti-m9D368 reactive p20–21 were not detected in ST-treated *caspase-3* (–/–) MEF cells (Figure 3), strongly suggesting that while p20–21 were the processing products of mcaspase-9 by activated mcaspase-3 via the feedback loop, activated mcaspase-3 preferentially processed mcaspase-9 at D³⁶⁸ and sites near the N-terminal rather than at D³⁵³. Anti-m9D368 reactive p20–21 were the major processing fragments of mcaspase-9 in ST-treated *WT* MEF cells, indicating that mcaspase-9 was initially autoprocessed at low levels at D³⁶⁸, and then later processed at this site by activated mcaspase-3 at higher levels.

That no Ac-DEVD-MCA cleavage activity was detected in ST-treated *caspase-9* (–/–) MEF cells (Figure 3A) showed that mcaspase-9 autoprocessing was necessary for mcaspase-3 activation. However, very low Ac-LEHD-MCA cleavage activity in ST-treated *caspase-3* (–/–) MEF cells strongly indicates that mcaspase-3 activation was necessary for the full activation of mcaspase-9. The disappearance of mcaspase-9 p20–21 fragments was consistent with the decreased Ac-LEHD-MCA cleavage activity, suggesting that the mcaspase-9 p20–21 fragments produced by activated mcaspase-3 in the feedback amplification loop played a predominant role in ST-mediated activation of mcaspase-9.

Interestingly, ST-treated *caspase-3* (–/–) MEF cells revealed apoptotic features with cell shrinkage despite having only low Ac-LEHD-MCA activity and no Ac-DEVD-MCA activity. This may be due to the activation of other caspases compensating for the loss of caspase-3 in *caspase-3* (–/–) MEF cells.²⁶

Anti-m9D168 reacted with p18 in ST-treated MEF cells but not in *caspase-9* (–/–) and *caspase-3* (–/–) MEF cells, suggesting that D¹⁶⁸ was one of the mcaspase-9 cleavage sites processed by mcaspase-3. Anti-m9D368 reacted with at least two small processing fragments in ST-treated *WT* MEF cells (Figure 4B), suggesting that mcaspase-9 was processed not only at D¹⁶⁸, but also at proximal sites. Unfortunately, anti-m9D168 could not be used for the immunohistochemical detection of caspase-9 processing fragments, perhaps due to the cellular distribution or conformational effects of caspase-9 processing fragments. Based on the molecular weights of the processing fragments, D¹⁷⁶ or D¹⁸⁸ could be considered as other candidate caspase-9 cleavage sites recognized by activated caspase-3. Characterization of these other caspase-9 cleavage sites is currently underway in our laboratory.

Processing of caspase-9 in the feedback amplification loop during programmed cell death of DRG neurons of mouse embryos

The very low level of p39-reacting anti-m9D368 and very weak immunochemical reactivity of anti-mD368 in ST-treated *caspase-3* (–/–) MEF cells (Figures 3B and 5A, f) suggested that anti-m9D368 immunostaining could be used for the detection of mcaspase-9 activation by activated mcaspase-3 via feedback amplification *in vivo*. *Caspase-3* (–/–) and *caspase-9* (–/–) mice are characterized by abnormal brain

development,^{11,12,27} and caspase-3 and caspase-9 activation is thought to play an essential role in programmed cell death during brain and peripheral nerve development. Our observation that anti-m3D175 reactivity was not detected in the DRG of *caspase-9* (–/–) mice (Figure 5B, k) supported the hypothesis that mcaspase-9 is necessary for the activation of mcaspase-3 in the programmed cell death of DRG during development. Furthermore, anti-m9D368 reactivity was undetectable in *caspase-3* (–/–) mice (Figure 5B, e), strongly suggesting that the mcaspase-9/-3 feedback loop also occurs in DRG programmed cell death during development.

Biological significance of the feedback amplification loop

Caspase-9 is autoprocessed by the Apaf-1/cytochrome *c* pathway, which in turn activates caspase-3 and downstream effectors. The CARD domains of caspase-9 and Apaf-1 interact to produce an apoptosome complex, in which caspase-9 plays a role in the recruitment of caspase-3 to the apoptosome complex²⁸ and the autoprocessed caspase-9 processes caspase-3 into its active form.^{8,9}

As the 177 amino acid CARD domain is located within the N-terminal region of mcaspase-9, processing of mcaspase-9 at D³⁶⁸ and D¹⁶⁸ results in the release of most of the CARD domain. Thus, through this feedback loop, fully activated caspase-9 lacking the CARD domain and caspase-3 are released from the apoptosome complex. Two observations about the activity of mature caspase-9 released from apoptosomes *in vitro* have been made. First, only caspase-9 in the apoptosome complex has caspase-9 activity, but not released mature caspase-9. Second, mature caspase-9 can have caspase-9 activity. In the former observation, caspase-9 processing by caspase-3 is thought to play a role in negative feedback resulting in decreased caspase-9 and caspase-3 activity. While in the latter observation, caspase-9 processing by the feedback loop is involved in positive feedback, that is, activation of caspase-9 and caspase-3 activity and the transportation of caspase-9 to target proteins localized in the cytoplasm and nuclei. This is supported by the finding that caspase-9 is redistributed and transported from mitochondria to the nucleus in the apoptotic neuronal cells.²⁹

The marked decrease in Ac-LEHD-MCA activity in ST-treated *caspase-3* (–/–) cells (Figure 3) suggested that mature caspase-9 released from apoptosomes by caspase-3 has caspase-9 activity *in vivo* and that caspase-9 processing by caspase-3 via a feedback loop plays an essential role in the full activation of caspase-9 stimulated by ST treatment.

p53- and *c-myc*-dependent apoptosis is inhibited in *caspase-9* (–/–) MEF cells,¹⁰ suggesting that caspase-9 is involved in the apoptotic pathway induced by these genes. However, caspase-9 processing is detected at a later stage of *c-myc*-induced apoptosis than caspase-3 processing, while cytochrome *c* release is detected at an earlier stage.³⁰ This led to speculation of an alternate apoptotic pathway in p53- or *c-myc*-dependent apoptosis. However, it may be possible to explain the conflicting

observations as the result of low level autoprocessing of caspase-9 at initial stages, but high level caspase-9 processing by activated caspase-3 during feedback amplification at later stages. Alternately, there may be initial processing of caspase-3, which in turn processes caspase-9 via the feedback loop, as caspase-9 can be weakly activated without prior autoprocessing.

Thus, activation of caspase-9 via a feedback amplification loop may play a crucial role in the various types of apoptosis and programmed cell death during development.

Materials and Methods

Cell culture

WT, *caspase-9* (−/−) and *caspase-3* (−/−) MEF cells^{11,27} were cultured in α -minimum essential medium (MEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO₂.

Ac-LEHD-MCA and Ac-DEVD-MCA cleavage activity

Ac-LEHD-MCA (caspase-9-like) and Ac-DEVD-MCA (caspase-3-like) cleavage activities were measured as described previously.^{21–23} After incubation, cells were washed twice with phosphate buffered saline (PBS), and the cell pellets lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. Cells were then centrifuged at 10 000 × *g* for 5 min. The cell extracts (50 μ g protein) were incubated with 100 μ M Ac-DEVD-MCA and Ac-LEHD-MCA (Peptide Institute, Osaka, Japan) in incubation buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol and 50 mM Tris-HCl pH 7.5, 10 mM dithiothreitol, respectively) at 37°C for 20 min. The reactions were stopped by the addition of 10% sodium dodecyl sulfate (SDS). Fluorescence intensity was measured at 380 nm for excitation and at 460 nm for emission.

Preparation of antisera against the cleavage sites of mcaspase-9

Antisera against cleavage sites of caspase-9 were prepared as described previously.¹⁹ Peptides corresponding to two C-terminal processing sites of mcaspase-9,¹⁵ DSEPD³⁵³ and LDQLD³⁶⁸, and putative cleavage site PRPVD¹⁶⁸ near the N-terminus (CDSEPD, CLDQLD and CPRPVD, respectively) were synthesized (Sawady Technology, Tokyo, Japan). Peptides were conjugated to keyhole limpet hemocyanin (KLH) and injected into rabbits, and antisera against the peptides generated. Anti-m9D353, anti-m9D368 and anti-m9D168 antibodies were purified by DSEPD-peptide, LDQLD-peptide and PRPVD-peptide affinity column chromatography, respectively.

Preparation of EGFP- or N-FLAG tagged activated caspases

cDNA fragments encoding mcaspase-9D353, mcaspase-9D368 and mcaspase-9D168 were amplified by polymerase chain reaction (PCR) using the following primers: forward primer for mcaspase-9D353 and mcaspase-9D368 and mcaspase-9D168: 5'-ATGGACGAGGCG-GACCGGCA G-3'; reverse primer for mcaspase-9D353: 5'-TCAATCTGGCTCAGAGTCACT-3'; reverse primer for mcaspase-9D368: 5'-CTAATCCAGCTCGTCCAAGGG-3'; and reverse primer for mcaspase-9D168: 5'-TCAGTCCACCGCCTGGGTG-3'. Amplification consisted of 1 cycle of 95°C for 2 min, 10 cycles of 95°C for 1 min and 60°C for 2 min, and 1 cycle at 60°C for 7 min. PCR products

were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), and then subcloned in-frame into the *EcoRI* site of the FLAG tagged (FLAG) CMV expression vector (Kodak, New Haven, CT, USA). FLAG-plasmids (5 μ g) were transfected into COS cells using the calcium-phosphate method.³¹ Cell extracts were used to examine the reactivities and specificities of anti-m9D353, anti-m9D368 and anti-m9D168 by immunoblot analysis.

Mouse caspase-8 and mcaspase-6 were isolated by screening a P19 EC cDNA library (Stratagene, LaJolla, CA, USA) using hcaspase-8 and hcaspase-6 cDNA fragments as probes. Nucleotide sequences of the isolated mcaspase-8 and mcaspase-6 cDNA were identical to that published previously.^{32,33} cDNA fragments encoding mcaspase-8 or cleaved fragment (mcaspase-8D387) lacking the Death Effector Domains (DED) were amplified by PCR using the following primers: mcaspase-8(-DED) forward primer: 5'-TCACGGACTTCAGACAAAG-3'; mcaspase-8(-DED) reverse primer: 5'-TTAGGGAGGGAAGAA-GAG-3'; and mcaspase-8D387(-DED) reverse primer: 5'-TCAATC-CACCTCTAAAGT-3'. Cleaved mcaspase-6 fragment (mcaspase-6D162) cDNA was amplified by PCR using the following primers: mcaspase-6D162 forward primer: 5'-ATGACAGAAACCGATGGCTT-3'; and reverse primer: 5'-TCAATCCACCATGTCCAG-3'. Cleaved caspase-2 fragment of rat (rcaspase-2D394).³⁴ cDNA was amplified by PCR using rcaspase-2D394 forward (5'-CTGGAAATGGCGCGT-CGA-3') and reverse (5'-CTAATCACTCTCCTACATCC-3') primers. PCR was carried out as described above and the PCR products cloned into the pGEM-T easy vector, and then subcloned in-frame into the *EcoRI* site of the FLAG-CMV expression vector. FLAG-tagged active forms of mcaspase-3 (mcaspase-3D175) and mcaspase-7 (mcaspase-7D198) were prepared as described previously.

The cDNA fragments encoding mcaspase-9 or cleaved fragments (mcaspase-9D353, mcaspase-9D368 and mcaspase-9D168) were also subcloned in-frame into the *EcoRI* site of the pEGFP-C1 vector (Clontech Lab. Inc., Palo Alto, CA). These EGFP- or FLAG-tagged cDNAs were transfected into COS cells and were subjected to immunostaining using anti-FLAG (Sigma, St. Louis, MO, USA), anti-m9D353, anti-m9D368 and anti-m9D168 antibodies.

Immunoblot analysis

Anti-m9D353, anti-m9D368, and anti-m9D168 reactivities were examined in apoptotic cells induced by ST (Sigma) and COS cells transfected with FLAG-mcaspase-9D353, -mcaspase-9D368 and mcaspase-9D168. After WT, *caspase-3* (−/−) and *caspase-9* (−/−) MEF cells were incubated with 1 μ M ST or COS cells transfected, cells were harvested at the indicated times and washed twice with PBS. Cell pellets were lysed in PBS containing 0.2% Triton X-100 for 10 min. After centrifugation at 10 000 × *g* for 10 min, the cell extracts (20 μ g protein) were subjected to SDS polyacrylamide (12%) gel electrophoresis. Proteins were transferred electrophoretically to nitrocellulose filters (SS, Dassel, Germany). Filters were incubated with monoclonal anti-PKC ϵ (Transduction Laboratories, Lexington, KY, USA), anti-m9D168, anti-m9D353, or anti-m9D368 reactivities were detected with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin (Promega) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate.

Immunochemical detection of the processing fragments of mcaspase-9

COS cells were transfected with pEGFP-mcaspase-9, mcaspase-9D353 or mcaspase-9D368 by the calcium-phosphate method. Cells were fixed at the indicated times with 4% paraformaldehyde in PBS at room temperature for 20 min, and incubated with anti-m9D353, anti-

m9D368 and anti-m9D168 for 24 h or 72 h at 4°C. Cells were then incubated with Texas Red-conjugated goat anti-rabbit immunoglobulin for 1 h at 37°C and viewed with a confocal laser scanning microscope (CSU-10, Yokokawa, Tokyo, Japan).

The ST-treated *WT*, *caspase-3* (–/–) and *caspase-9* (–/–) MEF cells were immunostained using anti-m3D175 and anti-m9D368 and immunoreactivities detected using a peroxidase-conjugated avidin-biotin kit (Vector Laboratories, Burlingame, CA, USA).

Immunohistochemical staining and terminal-deoxytransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)

WT, *caspase-3* (–/–) and *caspase-9* (–/–) mouse embryos at E11.5 were fixed with 4% paraformaldehyde in PBS at 4°C overnight and then soaked in 30% sucrose/PBS at 4°C overnight. Frozen sections (10 µm thick) were cut on a cryostat and attached to slides coated with VECTABOND reagent (Vector Laboratories). For double-staining, the sections were subjected to immunostaining using antisera against m9D368 and m3D175 and TUNEL-staining. Immunoreactivities were detected by FITC-conjugated goat anti-rabbit immunoglobulin (Biosource, Camarillo, CA, USA). To detect DNA fragmentation in cell nuclei, the TUNEL reaction was applied to fixed sections according to the modified method of Gavrieli *et al.*³⁵ Briefly, sections were incubated with 100 U/ml TdT (Boehringer Mannheim, Mannheim, Germany) and 10 µM biotinylated 16-2'-dUTP (Boehringer Mannheim) in a humid atmosphere at 37°C for 1 h. Further incubation with Texas Red-conjugated avidin (Seikagaku Co., Tokyo, Japan) was carried out for 1 h at room temperature. Signals were viewed with a confocal laser scanning microscope.

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