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Caspase-12 processing and fragment translocation into nuclei of tunicamycin-treated cells

E Fujita[1,4], Y Kouroku[1,4], A Jimbo[1], A Isoai[2], K Maruyama[3] and T Momoi*[,1]

[1] Divisions of Development and Differentiation, National Institute of Neuroscience, NCNP, Kodaira, Tokyo, Japan
[2] Asahi glass Co., Hasawa-cho, Kanagawa-ku, Yokohama, Kanagawa, Japan
[3] Department of Pharmacology, Saitama Medical School, Moroyama, Saitama, Japan
[4] The first two authors contributed equally to this work.
* Corresponding author: T Momoi, Division of Development and Differentiation, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187-8502, Japan. Tel: +81-42-341-2711 Ext 5273; Fax: +81-42-346-1754; E-mail: momoi@ncnp.go.jp

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Abstract

Excess endoplasmic reticulum (ER) stress induces processing of caspase-12, which is located in the ER, and cell death. However, little is known about the relationship between caspase-12 processing and cell death. We prepared antisera against putative caspase-12 cleavage sites (anti-m12D318 and anti-m12D341) and showed that overexpression of caspase-12 induced autoprocessing at D^{318} but did not induce cell death. Mutation analysis confirmed that D^{318} was a unique autoprocessing site. In contrast, tunicamycin, one of the ER stress stimuli, induced caspase-12 processing at the N-terminal region and the C-terminal region (both at D^{318} and D^{341}) and cell death. Anti-m12D318 and anti-m12D341 immunoreactivities were located in the ER of the tunicamycin-treated cells, and some immunoreactivities were located around and in the nuclei of the apoptotic cells. Thus, processing at the N-terminal region may be necessary for the translocation of processed caspase-12 into nuclei and cell death induced by ER stress. Some of the caspase-12 processed at the N-terminal and C-terminal regions may directly participate in the apoptotic events in nuclei.
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Keywords: caspase-12; ER stress; tunicamycin

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; TRAF2, TNF receptor-associated factor 2; JNK, c-Jun N-terminal kinase; CARD, caspase-associated recruit domain

Introduction

Unfolded or misfolded proteins in the endoplasmic reticulum (ER) trigger the unfolded protein response (UPR), which either improves local protein folding or results in cell death. Unfolded proteins activate stress signals via ER stress sensor proteins called IREs[1] that lead to the induction of ER stress. For instance, IRE1-α mediates ER stress signals induced by agents such as tunicamycin, which blocks N-linked protein glycosylation in the ER.[2] Upon activation, IRE1-α can recruit the cytosolic adaptor protein, TNF receptor-associated factor 2 (TRAF2), which in turn recruits and activates the proximal components of the c-Jun N-terminal kinase (JNK) pathway. ER stress also leads to the activation of genes possessing a UPR element in the promoter region.[3] Such genes include the Bip/Grp78 protein that increases protein folding in the ER lumen. When these stress signals are unable to rescue cells, the apoptotic pathway is activated. However, little is known about the molecular mechanism of cell death induced by ER stress.

Caspases are components of the apoptotic pathway in mammals[4] and are activated via sequential processing by caspase family members.[5] For instance, caspase-9, which has a caspase-associated recruit domain (CARD) located at the N-terminus, is activated in an apoptosome complex by association with Apaf-1 bound with cytochrome *c* via a CARD domain. Once activated, it then in turn activates downstream caspase-3.[6] Caspase-12, which also has a CARD domain and is specifically localized on the cytoplasmic side of the ER, is thought to play a role in ER stress-mediated cell death.[7] Caspase-12 is processed at amino acids D^{318} and D^{341}.[7–10] Several possible molecular mechanisms for the processing of caspase-12 have been postulated.[8–10] One is that caspase-12 is initially processed at the C-terminal region by calpain activated by ER stress, then activated and autoprocessed at D^{318}.[8] The other is that caspase-12 is released from TRAF2 complexes by ER stress and is then autoprocessed via homodimerization.[9] Caspase-12 is also processed at D^{94} by caspase-7 and then autoprocessed at D^{341}.[10] Thus, the molecular mechanism by which caspase-12 is activated by ER stress stimuli is not yet clear.

Antisera against the cleavage sites of caspases are useful for the detection of caspase processing and the intracellular localization of processed fragments.[11–13] Here we prepared antisera against putative caspase-12 cleavage sites, D^{341} and D^{318}, and examined the autoprocessing of caspase-12 by overexpression and the processing of caspase-12 induced by tunicamycin. Moreover, we also examined the intracellular localization of the processed fragments of caspase-12 induced by tunicamycin.

Here we showed that some of the processed fragments of caspase-12 induced by tunicamycin were localized in the nucleus.

Results

Immunoreactivity of putative cleavage sites of caspase-12

Antibodies against the putative caspase-12 cleavage sites, anti-m12D341 and anti-m12D318, specifically reacted with

46 kDa (FLAG-caspase-12D341) and 43 kDa (FLAG-caspase-12D318) fragments, respectively (Figure 1). Anti-m12D341 and anti-m12D318 did not react with the processed fragments of other caspases, including caspase-2, -3, -7, -8 and -9. Thus, anti-m12D341 and anti-m12D318 were specific for caspase-12-processed fragments at D^{341} and D^{318}, respectively.

Autoprocessing of caspase-12 by overexpression

When FLAG-caspase-12 was transfected into COS cells, the FLAG-caspase-12 fusion protein was processed into a 43 kDa fragment which corresponds to the molecular weight of FLAG-caspase-12D318 (Figure 2A). Anti-m12D318, which did not react with procaspase-12, reacted with the 43 kDa processed fragment of caspase-12, while anti-m12D341 did not. In contrast with FLAG-caspase-12, FLAG-caspase-12 with mutation at amino acid C^{298}, catalytic cysteine substituted for alanine [caspase-12(C298A)], was not processed (Figure 2B). Mutation of caspase-12 at amino acid D^{318} [caspase-12(D318A)] suppressed processing, whereas caspase-12(D341A) did not. Thus, FLAG-caspase-12 was only autoprocessed at D^{318} by overexpression.

Processing of caspase-12 by tunicamycin

Endogenous caspase-12 is processed at N-terminal and C-terminal sites by tunicamycin treatment.[7–9] Caspase-12 was highly expressed in C2C12 cells (mouse myoblast cells). Treatment of C2C12 cells with tunicamycin increased the level of Bip/Grp78 and led to the processing of caspase-12

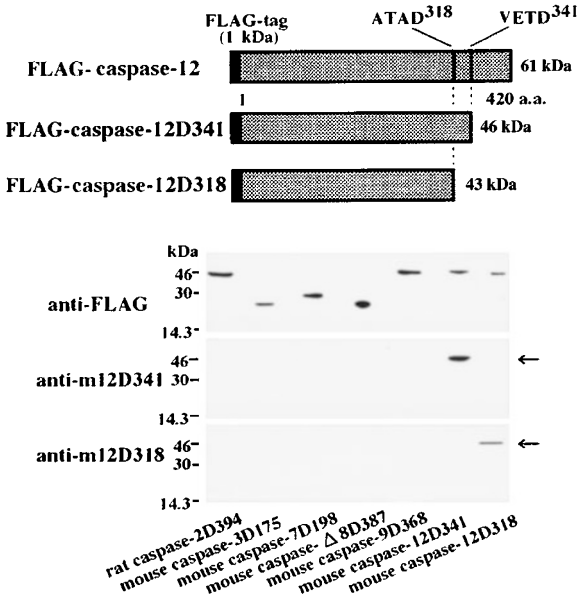

Figure 1 Preparation of anti-m12D341 and anti-m12D318 antibodies, antisera against the putative cleavage sites of caspase-12. Caspase-12 has two putative processing sites, D^{341} and D^{318}, at the C-terminal region. FLAG-caspase-12D341, -12D318, and FLAG-tagged other active caspases were transfected into COS cells, and the reactivities to anti-m12D341 and anti-m12D318 were examined by immunoblot analysis using anti-FLAG, anti-m12D341, and anti-m12D318

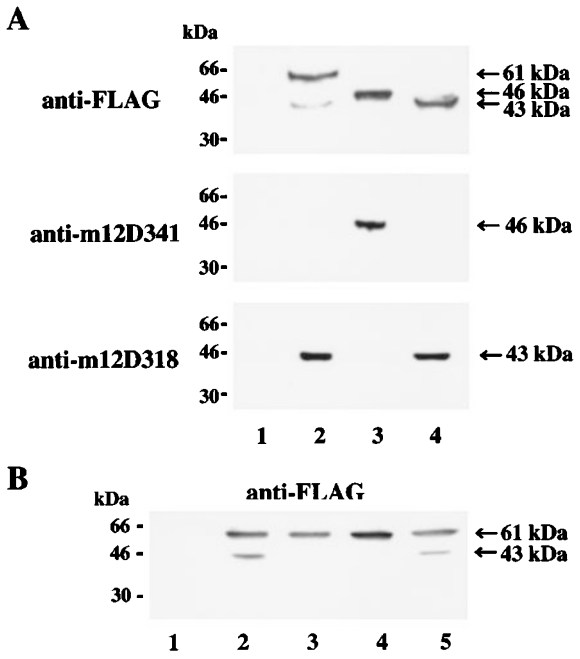

Figure 2 Autoprocessing site of caspase-12. (**A**) Immunoblot analysis of autoprocessing of caspase-12. FLAG-caspase-12, FLAG-caspase-12D341, and FLAG-caspase-12D318 were transfected into COS cells, and their expression was examined by immunoblot analysis using anti-FLAG, anti-m12D341, and anti-m12D318. Lane 1, untreated COS cells; lane 2, COS cells transfected with FLAG-caspase-12; lane 3, COS cells transfected with FLAG-caspase-12D341; lane 4, COS cells transfected with FLAG-caspase-12D318. (**B**) Effect of mutation of amino acid at cleavage sites on the processing of caspase-12. FLAG-caspase-12 with or without mutation was transfected into COS cells, and its processed fragments were examined by immunoblot analysis using anti-FLAG antibody. Lane 1, untreated COS cells; lane 2, COS cells transfected with FLAG-caspase-12; lane 3, COS cells transfected with FLAG-caspase-12(C298A); lane 4, COS cells transfected with FLAG-caspase-12(D318A); lane 5, COS cells transfected with FLAG-caspase-12(D341A)

into two major bands, 40 kDa and 55 kDa, and one minor band, 37 kDa (Figure 3). Anti-m12D341 reacted with the major band (40 kDa), while anti-m12D318 reacted with the minor band (37 kDa). However, the 55 kDa band did not react with anti-m12D341 and anti-m12D318. Other ER stress stimuli such as brefeldin A induced a similar processing pattern of caspase-12 (unpublished observation). Thus, endogenous caspase-12 was processed not only at D^{318}, but also at D^{341} by ER stress. Molecular sizes of processed fragments of caspase-12 at D^{341} and at D^{318} were smaller than FLAG-caspase-12D341 (46 kDa) and FLAG-caspase-12D318 (43 kDa), respectively, suggesting that, in addition to D^{341} and D^{318}, caspase-12 was processed at the N-terminal region by tunicamycin treatment.

The relationship between apoptosis and the processed fragments of caspase-12 induced by tunicamycin treatment

Overexpression of caspase-12 did not induce the death of C2C12 cells. We examined the effect of the autoprocessing of caspase-12 on cell death (Figure 4). Anti-m12D341 and anti-

m12D318 immunoreactivities were negative in the unstimulated C2C12 cells. When FLAG-caspase-12 was transfected into C2C12 cells, cells expressing FLAG-caspase-12 did not show anti-m12D341 and anti-m12D318 immunoreactivity in the initial time after transfection (Figure 4A, B, E and F insets). At 30 h after transfection, some of the cells overexpressing FLAG-caspase-12 showed anti-m12D318 immunoreactivity (Figure 4F), but did not show apoptotic features such as strong Hoechst 33342 staining and cell shrinkage (Figure 4G and H). In contrast, anti-m12D341 immunoreactivity was not detected in the cells overexpressing FLAG-caspase-12 (Figure 4B).

We examined the relationship between the processing of endogenous caspase-12 and cell death induced by tunicamycin. Anti-caspase-12 immunoreactivity co-localized with anti-KDEL immunoreactivity, a marker of ER, in the unstimulated cells (Figure 5A and B). Thus, procaspase-12 was specifically located in the ER of C2C12 cells as previously reported.[7] After tunicamycin treatment, anti-m12D341 immunoreactivity first appeared in the ER of non-apoptotic cells (Figure 5D, E and F). In the latter stage, most of the anti-m12D341 and anti-m12D318 immunoreactivities co-localized with anti-KDEL immunoreactivity (Figure 5G, H, J and K), but some was detected around and in the nuclei of the cells with apoptotic features (Figure 5H, I, K and L).

The localization of the processed fragment of caspase-12 at D^{341} in the nuclei was confirmed by the optical slice sectioning analysis (Figure 6). Anti-m12D341 immunoreactivities were detected in the nucleus labeled with propidium iodine (PI).

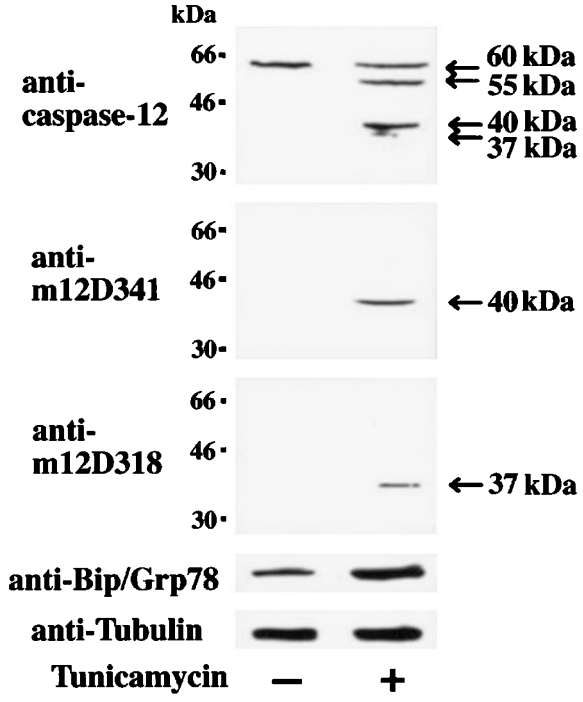

Figure 3 Processing of caspase-12 in C2C12 cells induced by tunicamycin treatment. C2C12 cells were treated with tunicamycin (1 μg/ml) for 30 h, and the processing of endogenous caspase-12 was examined by immunoblot analysis using anti-caspase-12, anti-m12D341, and anti-m12D318. The expression of Bip/Grp78 and tubulin was examined by immunoblot analysis using anti-Bip/Grp78 and anti-Tubulin, respectively

Discussion

Processing of caspase-12 by tunicamycin

It has been shown that caspase-12 is autoprocessed via internal and/or intramolecular systems following ER stress stimuli.[7–10] Anti-m12D341 and anti-m12D318 and mutation analysis showed that overexpression of caspase-12 induced autoprocessing at D^{318} (Figures 1 and 2). Upon ER stress, caspase-12 dissociates from TRAF2, forms a homodimeric complex, and is then autoprocessed.[9] Mouse caspase-9 is autoprocessed at D^{353} via association with Apaf-1 or

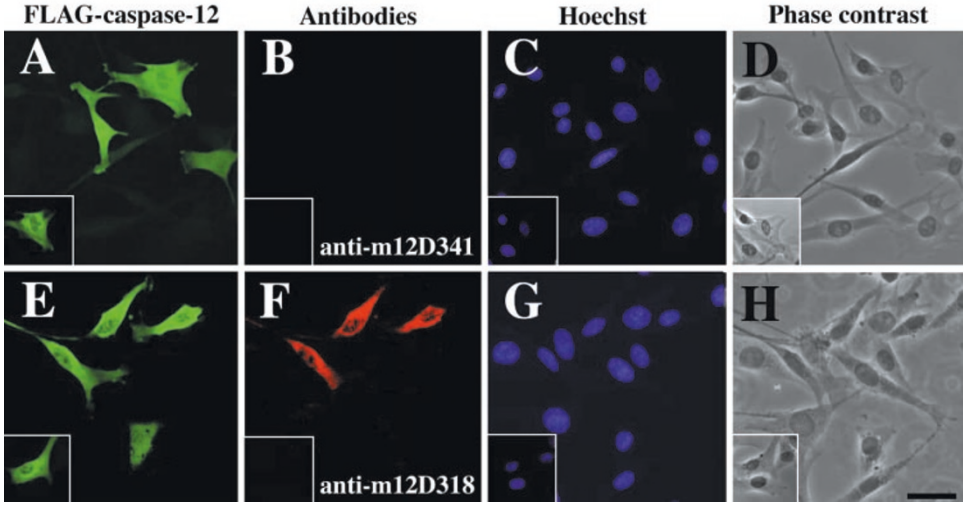

Figure 4 Anti-m12D341 and anti-m12D318 immunostaining analysis of the autoprocessing of FLAG-caspase-12. After FLAG-caspase-12 was transfected into C2C12 cells, anti-m12D341 (**A–D**) and anti-m12D318 (**E–H**) immunoreactivities were examined at 16 h (insets) and at 30 h after transfection. (**A** and **E**) FLAG-caspase-12; (**B**) anti-m12D341 immunostaining; (**F**) anti-m12D318 immunostaining; (**C** and **G**) Hoechst 33342 staining; (**D** and **H**) phase contrast. Bar=20 μm

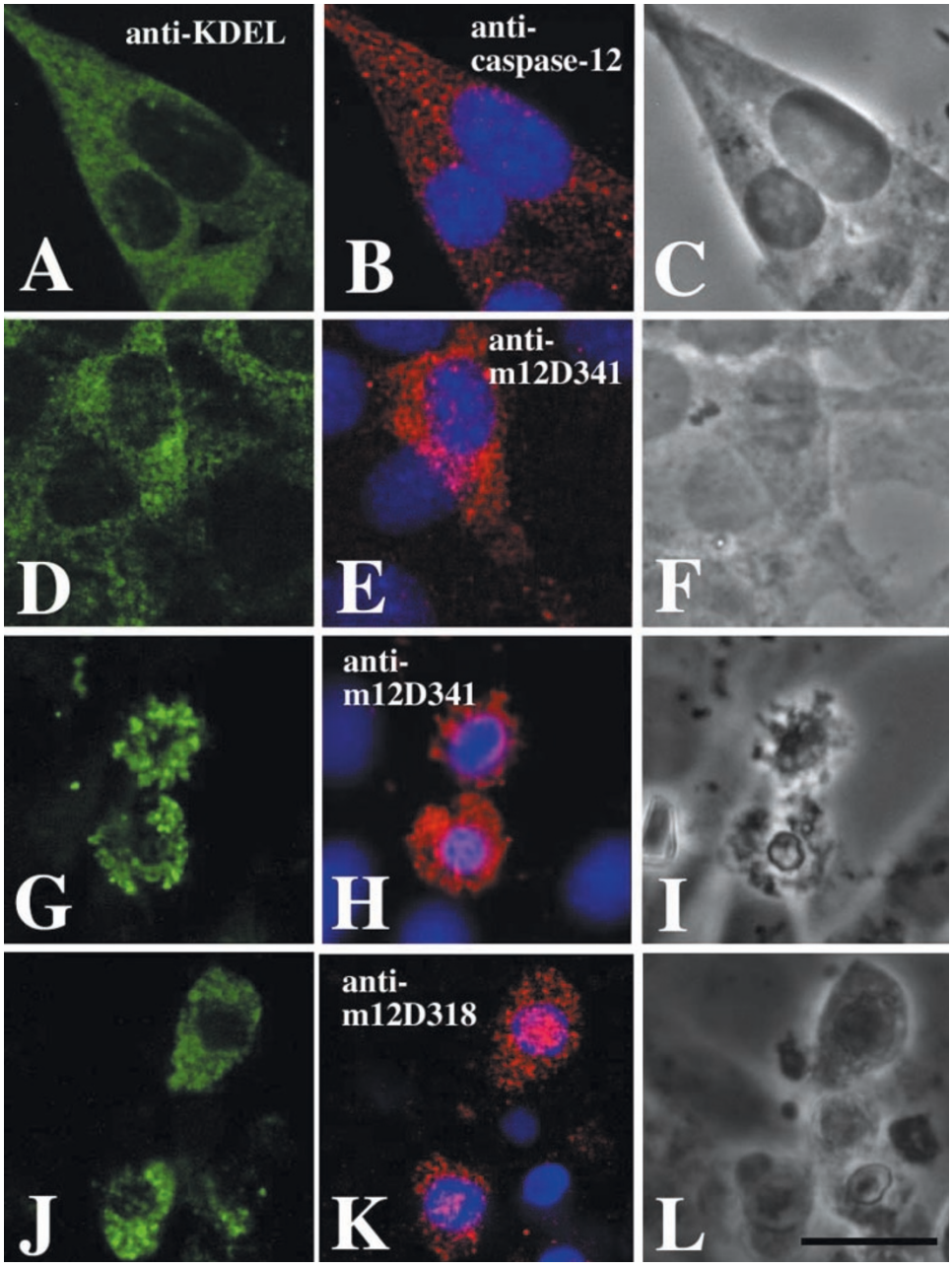

Figure 5 Localization of the processed fragments of caspase-12 in tunicamycin-treated C2C12 cells. Immunoreactivities of anti-m12D341 and anti-m12D318 in tunicamycin-treated C2C12 cells. Procaspase-12 and processed fragments of caspase-12 at D^{341} and D^{318} were examined by anti-caspase-12 (**B**) and anti-m12D341 (**E** and **H**) and anti-m12D318 antibodies (**K**). (**A–C**) unstimulated cells; (**D–L**) cells treated with tunicamycin (1 μg/ml) for 12 h (**D–F**) and for 30 h (**G–L**); (**A, D, G** and **J**) anti-KDEL staining (green); (**B, E, H** and **K**) double staining with anti-caspase-12 (**B**, red), anti-m12D341 (**E** and **H**; red), or anti-m12D318 (**K**, red) and Hoechst 33342 staining (blue); (**C, F, I** and **L**) phase contrast. Bar=20 μm

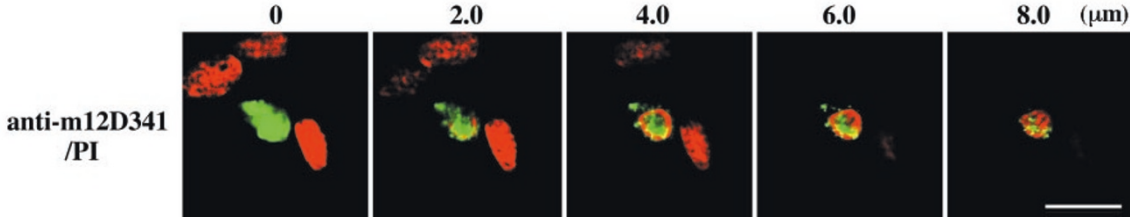

Figure 6 Detection of anti-m12D341 immunoreactivity in the nucleus by optical slice sectioning analysis. The optical slice section was carried out at 2.0 μm using a confocal laser scanning microscope. Anti-m12D341 immunoreactivities (green) were detected around and in the nucleus labeled with PI (red). Bar=20 μm

homodimerization through the CARD domain.[11] As caspase-12, along with caspase-2 and -9, contains an N-terminal CARD domain, it is possible that upon ER stress, caspase-12 may be autoprocessed at D^{318} through oligomerization or interaction with specific adapter molecules via the CARD domain.

In contrast with overexpression, however, caspase-12 was processed both at D^{341} and D^{318} in the C-terminal region by tunicamycin treatment (Figure 3). The C-terminal amino acid sequence of caspase-12 at D^{341}, VETD, is similar to the amino acid sequence of the processing site of mouse caspase-3 at D^{175}, IETD.[14] Human and mouse caspase-9 are first autoprocessed at D^{315} and D^{353}, respectively, and then processed at D^{330} and D^{368} by caspase-3 via a feedback-amplification loop, respectively.[11,15] It is possible that caspase-12 may be initially autoprocessed at D^{318} through oligomerization and then processed at D^{341} by downstream caspases in the feedback-amplification loop. However, as caspase-12 was not processed at D^{341} by overexpression (Figure 2), it is unlikely that the autoprocessed caspase-12 at D^{318} induces the processing at D^{341} via activation of downstream caspases.

The processing of caspase-12 at the N-terminal region

The molecular sizes of the processed fragments of caspase-12 induced by tunicamycin reacting with anti-m12D341 and anti-m12D318, 40 kDa and 37 kDa, suggested that in addition to D^{341} and D^{318}, caspase-12 was processed at the N-terminal region by tunicamycin treatment (Figure 3). The 55 kDa band, which did not react with anti-m12D341 and anti-m12D318, appears to be the large fragment of caspase-12 processed at the N-terminal region. It has been proposed that caspase-12 was processed by calpain and/or other caspases and then autoprocessed at D^{318} and at D^{341}, respectively.[8,10] However, as cleavage at the N-terminal site was not necessary for autoprocessing at D^{318} (Figure 2), the other explanation may be also possible: i.e., ER stress may activate other caspases as well as caspase-12 and cleave caspase-12 at D^{341} in parallel to the autoprocessing of caspase-12 at D^{318} or cleave caspase-12 at both D^{341} and D^{318}. These upstream caspases as well as calpain may cleave the N-terminal region of caspase-12 for activation. ER stress stimuli induce the activation of various caspases including caspases with Ac-DEVD-MCA cleavage activities (unpublished observation), suggesting this latter possibility. Further analysis of the activation of upstream caspases is currently underway in our laboratory.

The relationship between caspase-12 processing and cell death

Although ER stress induces the processing of caspase-12, little is known about the relationship between the activation of caspase-12 and the ER stress-mediated apoptotic pathway. Overexpression of caspase-12 induced autoprocessing at D^{318}, but anti-m12D318-positive cells induced by overexpression did not show apoptotic features (Figure 4). Thus, the D^{318} autoprocessing of caspase-12 is not sufficient for cell death.

The difference between the processing induced by overexpression and that induced by tunicamycin was the processing at D^{341} and N-terminal region (Figures 2 and 3). Caspases are processed at two sites in the C-terminal regions: i.e., mouse caspase-9 is processed at D^{353} and D^{368}.[11] It may be possible that differences in the processing at the C-terminal region cause differences in the activation of caspases. We do not exclude the possibility that processing at D^{341} is necessary for caspase-12 activation. On the other hand, processing at the N-terminal region itself is not necessary for the activation of caspases.[16]

The other major difference is the localization of the processed fragments of caspase-12. When caspase-12 was overexpressed, its autoprocessed fragments at D^{318} were localized in the cytoplasm (Figure 4); in contrast endogenous caspase-12 and its processed fragments at D^{341} and D^{318} induced by tunicamycin were initially localized in the ER (Figure 5). Thus, most of the exogenous caspase-12 overexpressed in the cytoplasm was autoprocessed by oligomerization probably via the CARD domain, whereas endogenous caspase-12 was initially processed in the ER upon tunicamycin treatment. We do not exclude the possibility that caspase-12 processed in the ER causes apoptosis via processing of the target molecule located in the ER.

However, we would like to address further the localization of anti-m12D341 and anti-m12D318 immunoreactivities in nuclei, which was not observed in the cells overexpressing FLAG-caspase-12 (Figure 4) but was detected in the apoptotic cells induced by tunicamycin (Figures 5 and 6). Caspase-9 is translocated into the nucleus from the cytoplasm after treatment with apoptotic stimuli.[17,18] Thus, it is possible that the processed fragments of caspase-12 in the ER may also be translocated into nuclei and participate in the apoptotic events in nuclei induced by ER stress. The lack of translocation of the autoprocessed fragment of caspase-12 into nuclei may not cause apoptosis of cells overexpressing FLAG-caspase-12.

What is the molecular mechanism by which the processed fragments of caspase-12 are translocated into nuclei in tunicamycin-treated cells? As caspase-12 has a CARD domain at the N-terminus, it is possible that endogenous caspase-12 may be associated with an anchor protein located on the cytoplasmic side of the ER via a CARD domain. Caspase-12 forms a complex with TRAF2,[9] and caspase-12 and TRAF2 co-localize in the ER (unpublished observation), suggesting that TRAF2 is one of the anchor proteins for caspase-12 on the ER membrane. The processing of caspase-12 at the N-terminal region, either by caspases or by calpain,[8] may cause the dissociation of caspase-12 from the ER. Furthermore, active nuclear transport of processed caspase-12 may be essential for apoptotic signal transduction.[19]

Activated caspase-12 induced by processing at the N-terminal region and the C-terminal region may be translocated into nuclei and participate in the apoptotic events in concert with other activated caspases. The elucidation of the molecular mechanisms of the activation

of other caspases and translocation of caspase-12 into nuclei will make clear the molecular mechanism of ER stress-mediated cell death.

Materials and Methods

Preparation of FLAG-fused caspase-12 and its processed fragments

The cDNA fragments encoding caspase-12 and its putative processed fragments at D^{341} (caspase-12D341) and at D^{318} (caspase-12D318) were amplified from RNA of C2C12 cells by reverse transcript-polymerase chain reaction (RT–PCR) using the following primers: forward primer for caspase-12, 5′-ATGGCGGCCAGGAGGACACATG-3′ and reverse primer for caspase-12, 5′-CTAATTCCCGG-GAAAAAGGTAG-3′; reverse primer for caspase-12D341, 5′-TCAATCTGTCTCCACATGGGC-3′; reverse primer for caspase-12D318, 5′-TCAATCAGCAGTGGCTATCC-3′. The cDNA fragments were amplified as follows: 1 cycle at 95°C for 2 min, 25 cycles at 95°C for 1 min and 60°C for 2 min, and 1 cycle at 60°C for 7 min. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and then subcloned in-frame into the *Eco*RI site of the pCMV-FLAG vector (Kodak, New Heaven, CT, USA). Nucleotide sequences were confirmed by dideoxy sequencing with a fully automated DNA sequencer, ALFII (Pharmacia, Milwaukee, WI, USA). FLAG-tagged processing fragments of other caspases such as rat caspase-2D169, mouse caspase-3D175, caspase-7D198, caspase-Δ8D387, and caspase-9D368 were prepared as described previously.[13]

Preparation of antisera against cleavage sites of caspase-12

Antisera against cleavage sites of mouse caspase-12 at D^{341} and D^{318}, anti-m12D341 and anti-m12D318, were prepared as described previously.[11–13] Briefly, peptides corresponding to a putative C-terminal processing site (D^{341} and D^{318}) of mouse caspase-12 and cysteine, $CHVETD^{341}$ and $CIATAD^{318}$, were synthesized (Sawady Technology, Tokyo, Japan). Anti-m12D341 and anti-m12D318 were generated by injecting CHVETD and CIATAD conjugated to keyhole limpet hemocyanin (KLH) into rabbits. Anti-m12D341 and anti-m12D318 antibodies were purified by CHVETD and CIATAD peptide affinity column chromatography, respectively.

Mutagenesis

We generated a mutant of caspase-12 by oligonucleotide-directed site-specific mutagenesis according to the method of Kunkel.[20] D^{341}, D^{318} and C^{298} were changed to A [caspase-12(D341A), caspase-12(D318A) and caspase-12(C298A)] by mutagenesis using 5′-pGTGGAGACAGCTTTCATTGC-3′, 5′-pCACTGCTGCTACAGATG-3′ and 5′-pATGCAGGCCGCCAGAGGCAG-3′ as primer, respectively. The mutation was verified by dideoxy sequencing.

Immunoblot analysis

COS cells and C2C12 cells were cultured in α-minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. pCMV-FLAG-plasmids (5 μg) were transfected into COS cells according to the calcium-phosphate method.[21] COS cells were washed twice with fresh medium 6 h after transfection and incubated for 30 h. C2C12 cells were treated with 1 μg/ml tunicamycin for 30 h. Cells were lysed with RIPA buffer (phosphate-buffered saline [PBS] containing 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). After centrifugation at 10 000 × *g* for 10 min, the cell extracts (50 μg protein) were subjected to SDS-polyacrylamide gel (12 %) electrophoresis and immunoblot analysis. Resolved proteins were electrophoretically transferred to nitrocellulose filters. After filters were incubated with anti-Bip/Grp78 (Stressgen Biotechnologies Corp., Victoria, BC, Canada), anti-Tubulin (Sigma), anti-m12D341, anti-m12D318, and anti-FLAG (Sigma) or anti-caspase-12 antibodies (Cell Signaling Technology, Beverly, MA, USA), the reactivities on the filters were detected by alkaline phosphatase-conjugated, goat anti-rabbit and anti-mouse immunoglobulin (Promega), respectively, and nitro blue tetrazolium and 5-bromo-4-chloro- 3-indolyl-1-phosphate.

Immunostaining

After C2C12 cells transfected with pCMV-FLAG-plasmids and treated with tunicamycin were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, they were incubated with anti-FLAG, anti-KDEL (Stressgen Biotechnologies Corp.) and anti-caspase-12, anti-m12D341, or anti-m12D318 for 30 h at 4°C. They were then incubated with FITC-conjugated, goat anti-mouse immunoglobulin or Texas Red-conjugated, goat anti-rabbit immunoglobulin for 1 h at 37°C, and apoptotic cells were labeled with Hoechst 33342 and viewed with a confocal laser scanning microscope (CSU-10, Yokokawa, Tokyo, Japan).

To detect the localization of the processed fragment of caspase-12 at D^{341} in the nucleus of the tunicamycin-treated cells, the optical slice section was carried out at 1.0 μm using a confocal laser scanning microscope. After cells were immunostained with anti-m12D341 and FITC-conjugated, goat anti-rabbit immnoglobulin, they were labeled with PI and then subjected to the optical slice sectioning analysis.

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