Cleavage of calnexin caused by apoptotic stimuli: Implication for the regulation of apoptosis

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Cleavage of Calnexin Caused by Apoptotic Stimuli: Implication for the Regulation of Apoptosis

Takenori Takizawa[1,*], Chizuru Tatematsu[1], Kimi Watanabe[1], Kanefusa Kato[1] and Yoshinobu Nakanishi[2]

[1]Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Kasugai 480-0392; and [2]Graduate School of Medical Science, Kanazawa University, Kanazawa 920-0934

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Calnexin is an endoplasmic reticulum (ER)-resident molecular chaperone that plays an essential role in the correct folding of membrane proteins. We found that calnexin is subjected to partial cleavage in apoptotic mouse cells. Both ER stress–inducing and ER stress–non-inducing apoptotic stimuli caused the cleavage of calnexin, indicating that this event does not always occur downstream of ER stress. The inhibition of caspases that target the amino acid sequence DXXD abrogated calnexin cleavage in apoptotic stimulus-treated cells. In addition, disruption of one of two DXXD sequences located in the cytoplasmic domain caused calnexin to escape cleavage during apoptosis. Furthermore, calnexin was cleaved *in vitro* by recombinant caspase-3 or caspase-7. Finally, the overexpression of a presumed cleavage product of calnexin partly inhibited apoptosis. These results collectively suggest that caspase-3 or caspase-7 cleaves calnexin, whose cleaved product leads to the attenuation of apoptosis.

Key words: apoptosis, calnexin, caspase, chaperone, ER stress.

Abbreviations: ER, endoplasmic reticulum; EGFP, enhanced green fluorescence protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The endoplasmic reticulum (ER) is the site of synthesis of membrane and secretory proteins, and regulates protein folding and trafficking, cellular responses to stress, and intracellular calcium (Ca^{2+}) levels (*1*). Alterations in Ca^{2+} homeostasis, the inhibition of N-linked glycosylation, and chemical toxicants, oxidative stress, *etc*. cause ER stress (*2*). When unfolded proteins accumulate and aggregate in the ER, a signal emerges that selectively activates the transcription of genes encoding ER chaperones to support correct protein folding, and inhibits protein synthesis to reduce the burden of protein substrates for ER-folding. This phenomenon is the so-called unfolded protein response (UPR) (*3*). Prolonged ER stress contributes to cell death and is linked to the pathogenesis of several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (*2*, *4*).

The apoptotic process involves the activation of caspases, a family of cysteine-dependent aspartate-directed proteases (*5*, *6*). The caspase family is broadly divided into two groups: initiator caspases (caspase-8, -9, -10) and effector caspases (caspase-3, -6, and -7) (*6*). Initiator caspases undergo autoprocessing for activation in response to apoptotic stimuli. Active initiator caspases in turn process precursors of effector caspases responsible for dismantling cellular structures. The localization of caspases and the translocation of their active products appear to be critical for the development of the apoptotic process (*6*). Caspase-12 has been shown to be specifically involved in apoptosis that results from stress in the ER (*7*, *8*). ER stress has been shown to result in the translocation of caspase-7 to the ER surface, where it cleaves caspase-12 (*9*). Prolonged ER stress results in the movement of active caspase-12 to the cytoplasm, where it interacts with caspase-9 (*9*).

Calnexin is a type I integral membrane protein with a large ER luminal domain, a transmembrane domain, and an ER-retention motif in its cytoplasmic tail (*10*). Calnexin is a molecular chaperone distributed mainly in the ER, that plays an essential role by interacting transiently with a diverse array of membrane proteins early in their synthesis (*11*). Calnexin promotes membrane protein assembly by retaining intermediates in the ER until the complete ternary complex is formed (*12*). In contrast, misfolded or incompletely assembled proteins show a prolonged association with calnexin.

In the present study, we show that several apoptotic stimuli cause the cleavage of calnexin in the carboxyl terminal region. Moreover, expression of the cleaved carboxyl terminal region of calnexin attenuates apoptosis, suggesting a novel mechanism of action during apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 and COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and maintained under 5% CO_2 at 37°C. Cells were transfected with 2 μg of plasmid DNA per 5×10^5 cells using 6 μl of Lipofectamine-plus and 4 μl of Lipofectamine (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. NIH3T3 cells were infected with influenza virus A/WSN/33 (H1N1) at a multiplicity of infection of 5 as described (*13*).

*To whom correspondence should be addressed. Tel: +81-568-88-0829 ext 3582, Fax: +81-568-88-0829, E-mail: takizawa@inst-hsc.jp

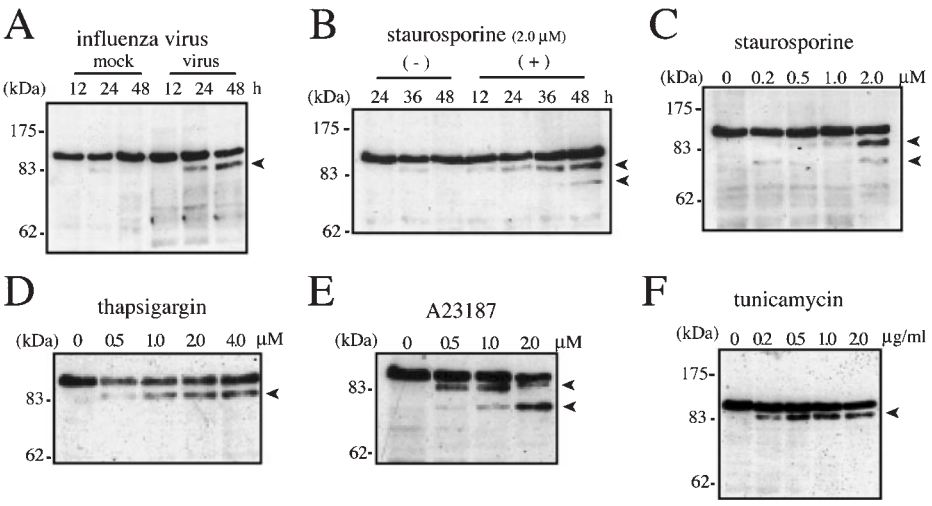

Fig. 1. **Cleavage of calnexin during apoptosis.** NIH3T3 cells incubated with the indicated reagents were lysed, and calnexin was detected by Western blotting. The term "mock" in A indicates that the cells were incubated with PBS containing no virus. Cells were incubated for 48 h in the presence of the reagents indicated in C–F. Cell lysates containing 20 μg of protein were analyzed in each lane. The arrowheads point to the positions of putative cleaved products of calnexin. The positions of molecular mass markers are indicated on the left.

Plasmids and DNA Transfection—Mouse calnexin cDNA was kindly provided by Dr. Larry W. Tjoelker (ICOS corporation, Bothell, WA, USA). Mutant calnexin cDNAs carrying a point mutation of D to E at position 527 or 568 were constructed by sequential polymerase chain reaction (PCR) as described previously (*14*). Primers used in this construction were as follows. *Pst*I/for: 5′-AGGTGTAGTGCTGCAGATGCT-3′. *Bam*HI/rev: 5′-GGGGATCC**TC-A**CTCTCTTCGTGGCTTTC-3′. DVKE/for: 5′-GATGTGAAGGA**A**GAAGAAGGGAAGGAA-3′. DVKE/rev: 5′-CCCTTCTTC**T**TCCTTCACATCTGGCTG-3′. DEEE/for: 5′-GATGAGGAAGA**A**AGCAAGCCTAAAGCA-3′. DEEE/rev: 5′-AGGCTTGCT**T**TCTTCCTCATCTTGACT-3′. XhoI/for: 5′-CCCTCGAGGAAGATAGCAAGCCTAAAGCA-3′. The primers *Pst*I/for, *Bam*HI/rev, and *Xho*I/for contain sites cleavable by the restriction enzymes *Pst*I, *Bam*HI and *Xho*I (underlined), respectively. *Bam*HI/rev has a stop codon before the *Bam*HI site (bold letters). The primers DVKE/for and DVKE/rev are complementary over 21 nucleotides, and contain one nucleotide change (bold letter) that creates an amino acid substitution of D to E at 527. The primers DEEE/for and DEEE/rev are also complementary over 21 nucleotides, and contain one nucleotide change (bold letter) that creates an amino acid substitution of D to E at 568. For the construction of DNA encoding the DVKE sequence, the upstream DNA fragment of DVKE was amplified using *Pst*I/for and DVKE/rev, and the downstream fragment was amplified with DVKE/for and *Bam*HI/rev. The resulting two DNA fragments were then annealed, and a secondary PCR was performed using *Pst*I/for and *Bam*HI/rev. For the construction of DNA encoding DEEE, the upstream fragment of DEEE was amplified using *Pst*I/for and DEEE/rev, and the downstream fragment was amplified with DEEE/for and *Bam*HI/rev. The resulting two DNA fragments were subjected to a secondary PCR as described above. These DNA fragments were digested with *Pst*I and *Bam*HI, and subcloned into *Pst*I/*Bam*HI–digested pEGFP-C3 to make fusion proteins with enhanced green fluorescence protein (EGFP) (Clontech Laboratories, Inc., Palo Alto, CA, USA). For the construction of the fusion protein containing the carboxyl terminal 45 amino acids, the above DNA fragment containing DEEE was digested with *Hin*dIII and *Bam*HI, and subcloned into *Hin*dIII/*Bam*HI–digested pEGFP-C3. For the construction of the fusion protein containing only the carboxyl terminal 25 amino acids, the DNA fragment was amplified using primers *Xho*I/for and *Bam*HI/rev, digested with *Xho*I and *Bam*HI, and subcloned into *Xho*I/*Bam*HI–digested pEGFP-C3. All the amplified DNA fragments were verified by DNA sequencing as described (*15*).

Indirect Immunofluorescence—Indirect immunofluorescence was performed as described (*16*). Briefly, cells were fixed with 4% paraformaldehyde containing 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 30 min and washed with PBS. Cells were then incubated with anti-CHOP polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a dilution of 100 for 60 min, stained with anti-rabbit immunoglobulin G antibody conjugated with fluorescein isothiocyanate (FITC) (MBL, Nagoya, Japan) at a dilution of 200 for 60 min, and observed under a fluorescence microscope at a magnification of 400 (Axiovert 200M, Carl Zeiss Light Microscopy, Gottingen, Germany). For the expression of EGFP, cells were fixed with 4% paraformaldehyde, and observed under a fluorescence microscope as described above.

Immunoblot Analyses—Cell extracts were prepared with Laemli's sample buffer (*17*) and boiled. Lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filters (Bio-Rad Laboratories, Hercules, CA, USA). Filters were incubated with anti-calnexin (StressGen Biotechnologies Corp., Victoria, Canada) or anti-GFP polyclonal antibody (Santa Cruz Biotechnologies) at a dilution of 1,000 for 60 min followed by anti-rabbit immunoglobulin antibody conjugated with peroxidase at a dilution of 1,000 for 60 min. Signals were visualized using an ECL detection system (Amersham Biosciences K.K., Tokyo, Japan).

In Vitro Cleavage of Calnexin—Mouse calnexin cDNA was transcribed and translated *in vitro* using a TNT kit (Promega Corporation, Madison, WI, USA) in the presence of ^{35}S-methionine (ICN Biomedicals, Inc., Costa Mesa, CA, USA). *In vitro* synthesized calnexin was then incubated with active recombinant caspases-Group II (MBL) in a reaction mixture containing 50 mM HEPES,

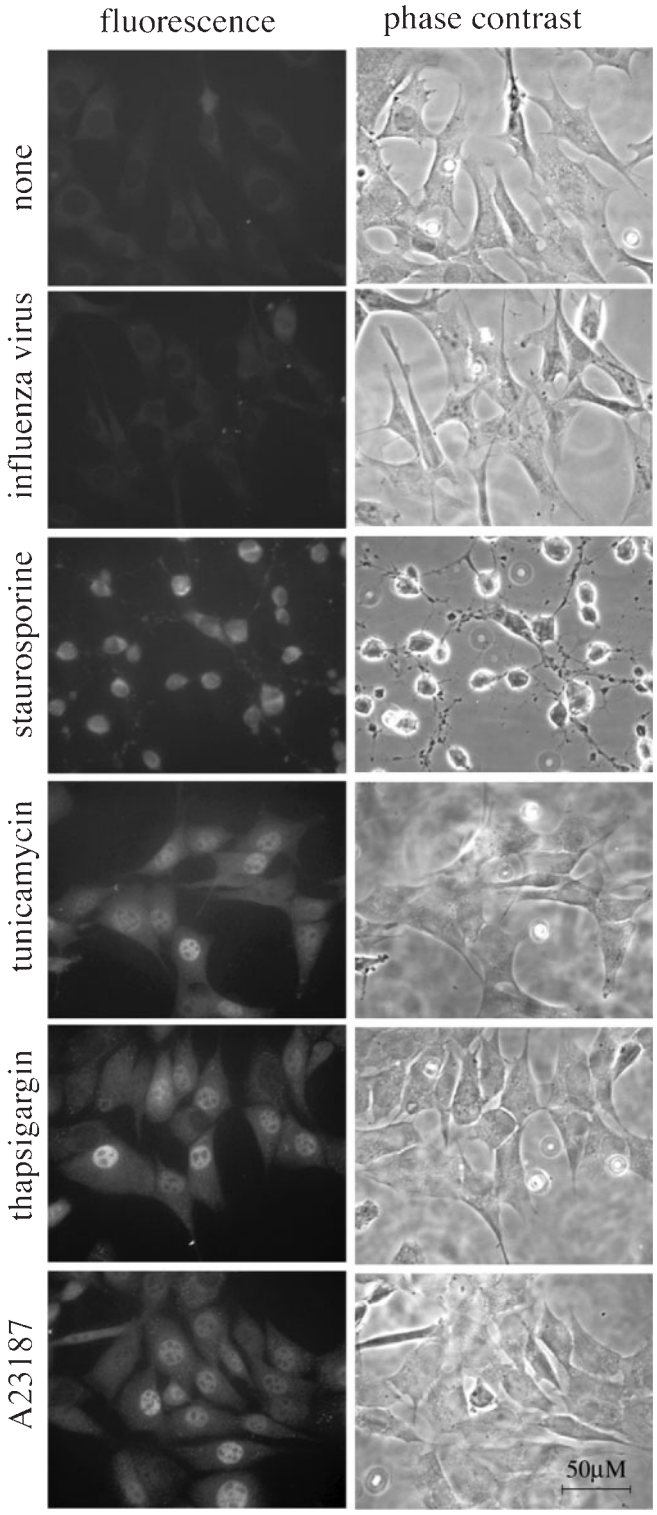

Fig. 2. **CHOP expression in cells treated with inducers of calnexin cleavage.** NIH3T3 cells were exposed to the indicated reagents for 8 h and analyzed for the expression of CHOP by immunofluorescence microscopy. Fluorescence and phase contrast images of the same microscopic fields are shown at a magnification of 400.

pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% glycerol, and 10 mM dithiothreitol for 6 h at 37°C. Reactions were resolved by SDS-PAGE followed by fluorography.

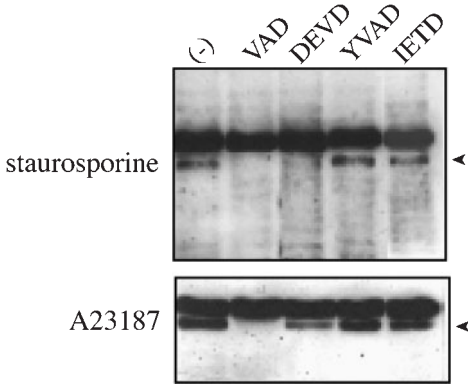

Fig. 3. **DEVDase-mediated cleavage of calnexin.** NIH3T3 cells were exposed to staurosporine (2 μM) or A23187 (2 μM) in the absence (–) or presence of z-VAD-fmk (VAD, 40 μM), z-DEVD-fmk (DEVD, 40 μM), Ac-YVAD-CHO (YVAD, 100 μM), or z-IETD-fmk (IETD, 40 μM) for 48 h, and then lysates containing 20 μg of protein were examined by western blotting with anti-calnexin antibody. The arrowheads denote the position of a cleaved fragment of calnexin.

RESULTS

Apoptotic Stimuli Induce the Cleavage of Calnexin in Cells—During an analysis of influenza virus–induced apoptosis (*18*), we found that the ER-resident molecular chaperone calnexin appeared to be degraded (Fig. 1A). To investigate this event further, we examined several inducers of apoptosis and ER stress for their effect on calnexin degradation. All the reagents tested induced apoptosis in NIH3T3 cells as assessed by the occurrence of DNA fragmentation (data not shown), and, at the same time, faster migrating signals became detectable with anti-calnexin antibodies (Fig. 1, B–F). The appearance of these signals depended on the duration of incubation and the dose of inducers. Treatment with influenza virus, thapsigargin (a Ca^{2+}-dependent ATPase inhibitor), or tunicamycin (an inhibitor of glycosylation in the ER) gave single cleaved products, while treatment with staurosporine (protein kinase C inhibitor) or A23187 (calcium ionophore) gave two products. These results indicate that calnexin is cleaved during apoptosis as well as under ER stress.

Since ER stress has been shown to induce the expression of the transcription factor C/EBP-homologue protein (CHOP) (*19*), we examined the effect of the above stimuli on CHOP expression using immunofluorescence microscopy (Fig. 2). Untreated NIH3T3 cells were negative for CHOP expression, whereas treatment with tunicamycin brought about fluorescence signals located in the nucleus, indicating that CHOP expression is induced by ER stress. The expression of CHOP was similarly induced by treatment with thapsigargin or A23187, but this was not the case in staurosporine-treated or influenza virus–infected cells. Although staurosporine-treated cells showed intense staining of the cytoplasm due to cell rounding caused by this reagent, CHOP expression was not observed by Western blotting (data not shown). These results indicate that ER stress, at least that accompanied by CHOP expression, is not always prerequisite to the cleavage of calnexin.

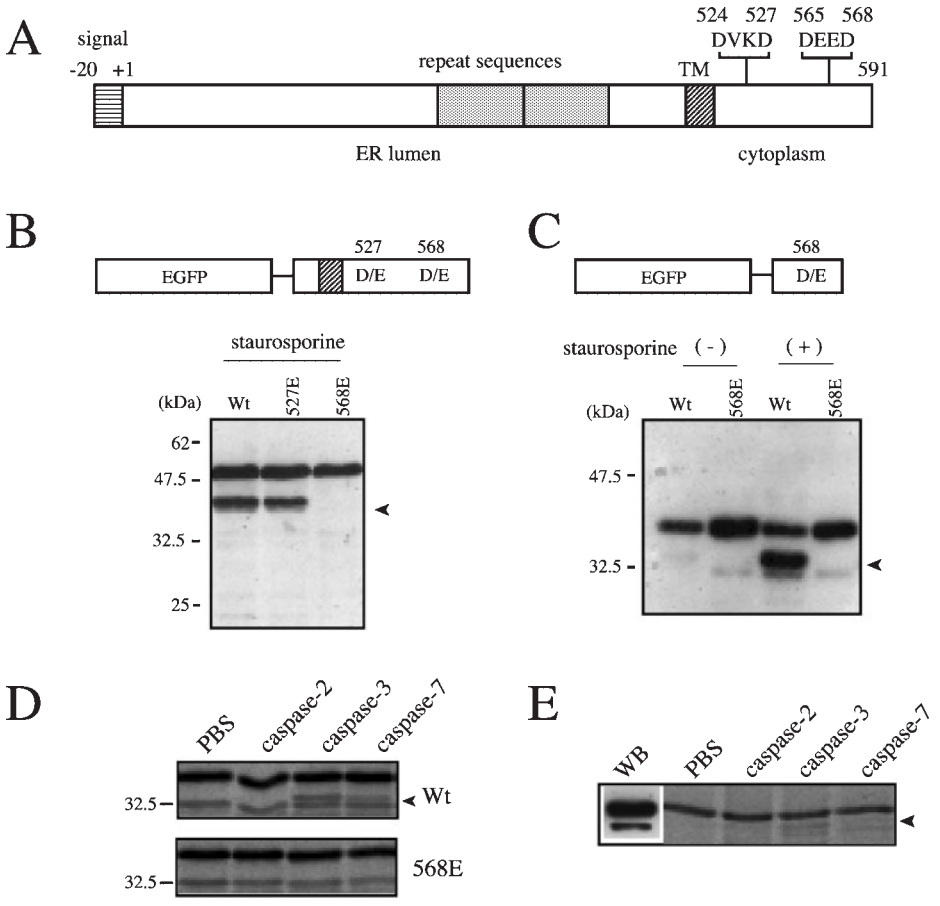

Fig. 4. **Identification of the cleavage site of mouse calnexin.** (A) Schematic representation of mouse calnexin. Two sequences identical to the consensus sequence for the group II caspase, DEVDases are shown with amino acid positions. (B) Requirement of the DEED sequence for calnexin cleavage. EGFP fused to a carboxyl terminal region of calnexin with or without alteration of the DXXD sequence was expressed in COS-1 cells, and then lysates containing 20 μg of protein after staurosporine treatment for 48 h were analyzed by immunoblotting. (C) Cleavage at the DEED sequence. EGFP fused to a portion of calnexin including only the DEED sequence was expressed in COS-1 cells and analyzed as in B. (D and E) Cleavage by caspases. EGFP-fused proteins (as described in C) (D), or an entire region of mouse calnexin (E), were synthesized *in vitro*, treated with the indicated active caspases, and separated by SDS-PAGE. A fluorogram of the gel is shown. A lysate of staurosporine-treated cells was simultaneously separated by SDS-PAGE and analyzed by Western blotting (WB).

Caspases Cleave Calnexin—To assess whether caspases are involved in the cleavage of calnexin, the effect of caspase inhibitors was examined (Fig. 3). The pan-caspase inhibitor, z-VAD-fmk, almost completely inhibited calnexin cleavage induced by all the stimuli examined above; the results with staurosporine and A23187 are shown as examples. Another inhibitor, z-DEVD-fmk, also inhibited the cleavage by staurosporine or A23187, whereas Ac-YVAD-CHO and z-IETD-fmk had no effect. Therefore, the activity of caspases, DEVDase in particular, is required, either directly or indirectly, for the cleavage caused by those stimuli.

Since the cytoplasmic domain of mouse calnexin contains two amino-acid sequences identical to the consensus sequence of group II caspases, DXXD (*20*), we anticipated that one or both of the sequences are the sites for caspase-mediated cleavage. To examine this possibility, we expressed EGFP fused to the carboxyl terminal region containing both sequences in COS-1 cells and determined the size of the fusion protein after staurosporine treatment by immunoblotting using anti-GFP antibody. We found that a faster migrating signal was present in cell lysates together with a presumed full-length protein (Fig. 4B). When a fusion protein in which one of the two sequences was disrupted was expressed, the protein with the altered sequence nearer the carboxyl terminal was no longer cleaved (Fig. 4B). To confirm this result, EGFP fused to 45 amino acids of the carboxyl terminal region containing only this sequence was tested. The fused protein expressed in COS-1 cells was cleaved upon staurosporine treatment, and a D to E alteration of this sequence abolished the cleavage (Fig. 4C). The same results were obtained when cells were exposed to tunicamycin or thapsigargin (data not shown). Furthermore, the fused protein was *in vitro* translated, and examined for whether caspases directly cleave the putative target sequence. Caspase-3 or caspase-7, even though to a lesser extent, cleaved the fused protein but not that with a D to E alteration (Fig. 4D). Full-length calnexin synthesized *in vitro* was also directly cleaved by caspase-3 (Fig. 4E). A very faint band could be observed in the case of caspase-7. The caspase-3 and caspase-7 used in this study cleaved a synthetic luminogenic substrate containing a DEVD sequence to the same extent (data not shown), suggesting that the different cleavability of calnexin is due to different substrate specificities. These results indicate that mouse calnexin is cleaved at 568D directly by a DEVDase-like caspase(s) in apoptotic cells.

Cleaved Calnexin Attenuates Apoptosis—Since the amount of endogenous calnexin in the intact form is much greater than that of the cleaved form even in cells exposed to apoptotic stimuli, it is very difficult to see the effect of the endogenous cleaved product on apoptosis directly. Therefore, we employed EGFP fused to the presumed cleaved fragment of calnexin between amino acids 567 and 591 to assess the biological role of the cleavage. The calnexin-EFGP fusion protein was expressed in NIH3T3 cells, and the extent of apoptosis in the cells was

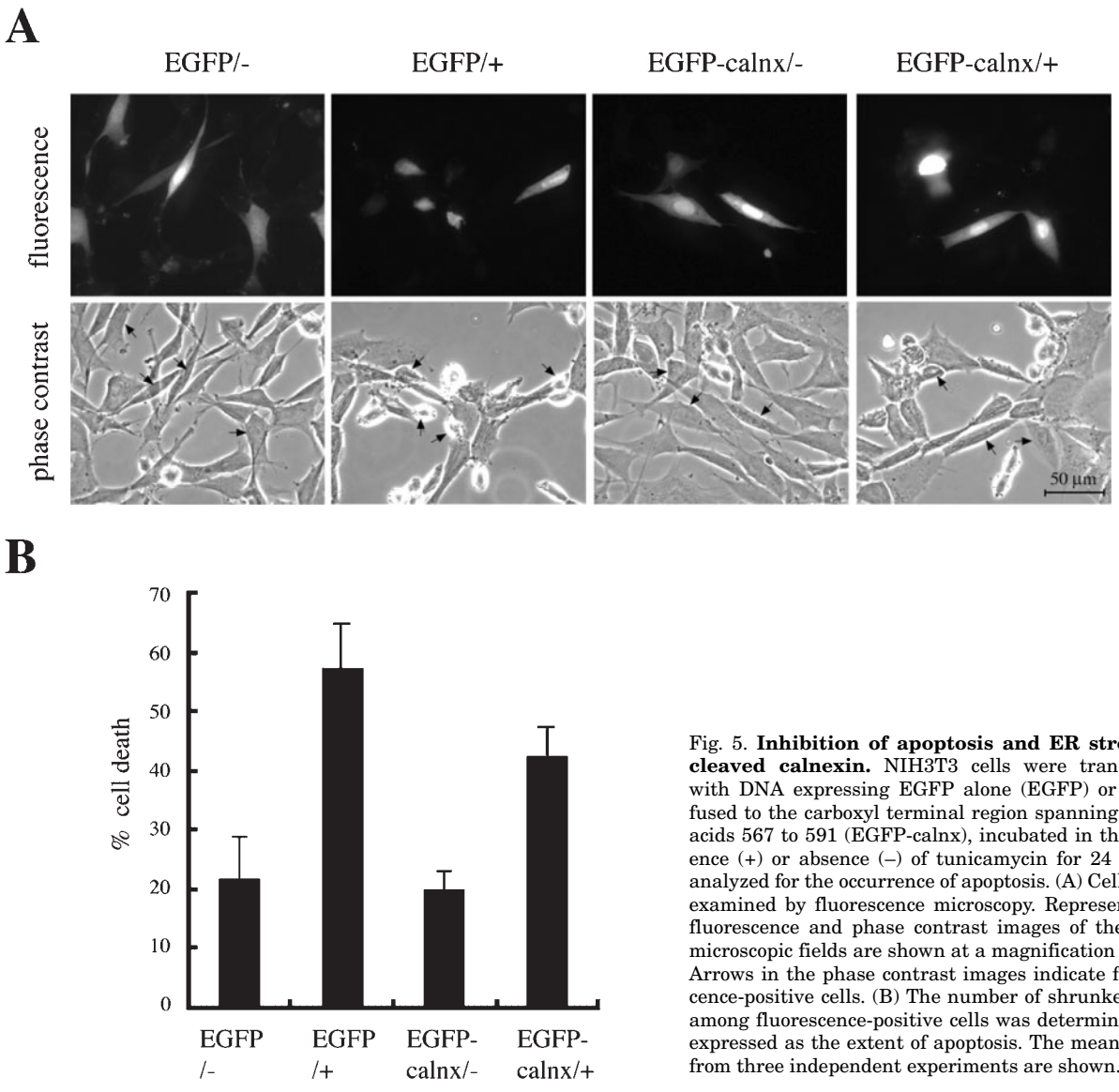

Fig. 5. **Inhibition of apoptosis and ER stress by cleaved calnexin.** NIH3T3 cells were transfected with DNA expressing EGFP alone (EGFP) or EGFP fused to the carboxyl terminal region spanning amino acids 567 to 591 (EGFP-calnx), incubated in the presence (+) or absence (–) of tunicamycin for 24 h, and analyzed for the occurrence of apoptosis. (A) Cells were examined by fluorescence microscopy. Representative fluorescence and phase contrast images of the same microscopic fields are shown at a magnification of 400. Arrows in the phase contrast images indicate fluorescence-positive cells. (B) The number of shrunken cells among fluorescence-positive cells was determined and expressed as the extent of apoptosis. The means ± SD from three independent experiments are shown.

examined after treatment with tunicamycin (Fig. 5). Most cells expressing EGFP alone appeared shrunken, rounded and apoptotic following exposure to tunicamycin. However, these changes in cells expressing the cleaved calnexin were reduced to about 60% of those in cells expressing EGFP alone, considering that the proportion of spontaneously dying cells without the reagent was about 20% (Fig. 5B). In contrast, preliminary results indicated that the fused protein did not inhibit staurosporine-induced morphological changes (data not shown). These results suggest that the cleaved product of calnexin attenuates, at least, ER stress–induced apoptosis.

We next examined the expression of the transcription factor CHOP in the same cells by immunoblotting. As observed above (Fig. 2), tunicamycin treatment induced the expression of CHOP, and this was partly inhibited when the cleaved product of calnexin was present (about 60% of control as assessed by densitometry) (Fig. 6). This effect of the cleaved product on CHOP expression may be underestimated since the efficiency of the transient transfection was about 30% or less. Essentially the same results were observed when cells were exposed to thapsigargin or A23187 (data not shown).

DISCUSSION

ER stress leads to the induction of apoptosis via the activation of caspase-12 (*7*, *8*). Caspase-12 could thus be a candidate for the enzyme that cleaves calnexin. However, our study showed that the sequence DEED located near the carboxyl terminus of calnexin is most likely to be the site of cleavage, and this sequence does not match the target sequence of caspase-12. The sequence DEED could be targeted by group II caspases (*20*), and, indeed, recombinant caspase-3, and probably caspase-7 as well, directly proteolysed calnexin *in vitro*. In addition, calnexin cleavage was observed in cells undergoing apoptosis not accompanied by ER stress. Collectively, it is most likely that calnexin is subjected to proteolysis at a site near the carboxyl terminus by caspase-3 or caspase-7 in

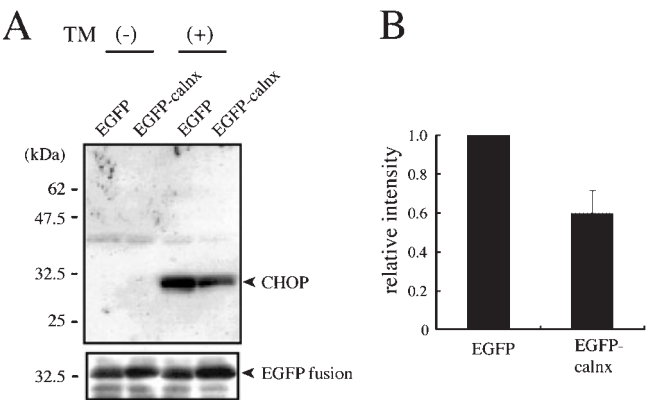

Fig. 6. **Inhibition of CHOP expression by cleaved calnexin.** NIH3T3 cells were transfected with DNA expressing EGFP alone (EGFP) or EGFP fused to the carboxyl terminal region (EGFP-calnx) as indicated in Fig. 5, and incubated in the presence (+) or absence (–) of tunicamycin for 8 h. (A) Cells were lysed and analyzed for the expression of CHOP and EGFP-fusion proteins by immunoblotting. The arrowheads indicate the positions of CHOP and EGFP-fusion proteins. (B) The intensity of CHOP signals was determined, and that in lysates with the fusion protein is shown relative to that with EGFP alone. The means ± SD from three independent experiments are shown.

apoptotic cells. Caspase-2, however, may not cleave this site, since the preferential peptide substrates of caspase-2 are different from those of caspase-3 and caspase-7 (*5*). Calnexin was cleaved into smaller fragments upon exposure to staurosporine or A23187 (Fig. 1), indicating the existence of an additional cleavage site. Judging from the size of the smaller fragment, the site seems to exist in the ER luminal domain. Since there is no sequence that matches the caspase cleavage sites in the ER luminal domain (*10*), caspases are unlikely to cleave this site. Further analysis is required to clarify this issue.

The mechanism by which the cleaved product of calnexin inhibits apoptosis, if it occurs at all, remains to be clarified. Our data indicate that the expression of the transcription factor CHOP, which is induced by ER stress (*19*), was inhibited in cells upon overexpression of a presumed cleavage product of calnexin. It is thus possible that calnexin-mediated attenuation of apoptosis is accompanied by the inhibition of CHOP expression. However, this cannot be the general mechanism since calnexin cleavage occurs in apoptotic cells with no evidence of a response to ER stress. Moreover, while the induction of CHOP begins early (within 8 h after ER stress), calnexin cleavage occurs relatively late in apoptosis. Therefore, it seems likely that the cleaved product of calnexin does not inhibit the expression of CHOP directly, but rather suppresses it indirectly by reducing apoptosis during an ER stress.

In contrast, calnexin-deficient cells are relatively resistant to ER stress–induced apoptosis (*21*). It has been explained that the cleavage of Bap31, whose product p20 induces apoptosis, is inhibited in calnexin-deficient cells, since the cytoplasmic tail of calnexin may play a role in caspase 8–dependent cleavage of Bap31 (*21*). It is thus conceivable that the cytoplasmic tail of calnexin may mediate the Bap31-calnexin interaction and the cleavage of the cytoplasmic tail might reduce the generation of p20 with a resultant attenuation of Bap31-dependent apoptosis. Overexpression of the EGFP-calnexin tail might compete for the Bap31-calnexin interaction. Further analyses are required to clarify this hypothesis. That calnexin lacking an ER retention signal changes its subcellular localization to the Golgi apparatus or the cell surface (*22*) suggests an unexpected mechanism for the calnexin-mediated inhibition of apoptosis.

Another ER chaperone, calreticulin, has been suggested to induce apoptosis (*23*) and to be involved in the recognition of apoptotic cells by phagocytes (*24*). Although it is not known if calreticulin undergoes proteolysis and a change in subcellular localization during apoptosis, ER chaperones thus could be generally involved in apoptotic events.

In summary, the findings obtained in this study lead us to propose a novel mechanism for the control of apoptosis: the cytoplasmic domain of the ER chaperone calnexin is liberated by caspases in apoptosing cells and acts to attenuate apoptosis. Further studies are needed to confirm this issue.

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