POSTTRANSLATIONAL N-MYRISTOYLATION IS REQUIRED FOR THE ANTI-APOPTOTIC ACTIVITY OF HUMAN tGELSOLIN, THE C-TERMINAL CASPASE-CLEAVAGE PRODUCT OF HUMAN GELSOLIN

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Protein N-myristoylation has been recognized as a cotranslational protein modification. Recently, it was demonstrated that protein N-myristoylation could occur posttranslationally, as in the case of the pro-apoptotic protein BID and cytoskeletal actin. Our previous study showed that the N-terminal 9 residues of the C-terminal caspase-cleavage product of human gelsolin, an actin-regulatory protein, efficiently direct the protein N-myristoylation.

In this study, to analyze the posttranslational N-myristoylation of gelsolin during apoptosis, metabolic labeling of gelsolin and its caspase-cleavage products expressed in COS-1 cells with [3H]myristic acid was performed. It was found that the C-terminal caspase-cleavage product of human gelsolin (tGelsolin) was efficiently N-myristoylated. When COS-1 cells transiently transfected with gelsolin cDNA were treated with etoposide or staurosporine, apoptosis-inducing agents, N-myristoylated tGelsolin was generated, as demonstrated by in vivo metabolic labeling. The generation of posttranslationally N-myristoylated tGelsolin during apoptosis was also observed on endogenous gelsolin expressed in Hela cells. Immunofluorescence staining and subcellular fractionation experiment revealed that exogenously expressed tGelsolin did not localize to mitochondria, but rather was diffusely distributed in the cytoplasm.

To study the role of this modification in the anti-apoptotic activity of tGelsolin, we constructed the bicistronic expression plasmid tGelsolin-IRES-EGFP capable of overexpressing tGelsolin concomitantly with EGFP. Overexpression of N-myristoylated tGelsolin in COS-1 cells using this plasmid significantly inhibited etoposide-induced apoptosis, whereas overexpression of the non-myristoylated tGelsolinG2A mutant did not cause resistance to apoptosis.

These results indicate that posttranslational N-myristoylation of tGelsolin does not direct mitochondrial targeting, but this modification is involved in the anti-apoptotic activity of tGelsolin.

INTRODUCTION

Protein N-myristoylation is a well-recognized form of lipid modification that occurs on eukaryotic and viral proteins (1-5). Many N-myristoylated proteins play critical roles in regulating cellular structure and function. They include proteins involved in a wide variety of cellular signal transduction pathways such as protein kinases, phosphatases, guanine nucleotide-binding proteins, and Ca^{2+}-binding proteins. In many cases, the functions of these N-myristoylated proteins are regulated by reversible protein-membrane and protein-protein interactions mediated by protein N-myristoylation. Generally, protein N-myristoylation is the result of cotranslational attachment of myristic acid, a 14-carbon saturated fatty acid, to a Gly residue at the extreme N-terminus.
after removal of the initiating Met. A stable amide bond links myristic acid irreversibly to proteins. In addition to the cotranslational protein N-myristoylation, it was demonstrated that posttranslational protein N-myristoylation can also occur, as in the case of the pro-apoptotic protein BID. In this case, proteolytic cleavage of BID by caspase 8 caused exposure of an internal N-myristoylation motif (6). The exposed internal N-myristoylation motif was recognized by N-myristoyltransferase (NMT), the enzyme responsible for cotranslational N-myristoylation, and posttranslational N-myristoylation reaction occurred. It was also revealed that this post-proteolytic N-myristoylation of BID plays critical role in the targeting of BID to mitochondria, its insertion into the outer membrane of mitochondria, the release of cytochrome c, and the killing of cells. Thus, posttranslational N-myristoylation plays a crucial role in the biological activity of BID.

Until recently, BID was the only protein that had been demonstrated to be posttranslationally N-myristoylated. However, we recently showed that the C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and specifically targeted to mitochondria (7). In this case, tActin localized at mitochondria did not induce cellular apoptosis. The biological roles of the mitochondrial localization of tActin remain to be clarified. During the analysis of posttranslational N-myristoylation of tActin, we also found that the N-terminal nine residues of the newly exposed N-terminus of the caspase-cleavage product of human gelsolin, an actin-regulatory protein, efficiently direct the protein N-myristoylation. Gelsolin is a member of a large family of actin-severing and -capping proteins (8, 9). Human gelsolin has been shown to inhibit apoptosis through its ability to block the loss of mitochondrial membrane potential and to inhibit caspase activity (10-14). The role of gelsolin in apoptosis is complicated by the fact that gelsolin is also a substrate for caspase-3, caspase-7 and caspase-9, which generate two cleavage products with opposite function, an N-terminal fragment with pro-apoptotic activity and a C-terminal fragment with anti-apoptotic activity (12, 14-16). The precise mechanisms by which the two fragments affect cellular apoptosis remain to be elucidated.

In the present study, the posttranslational N-myristoylation of the caspase-cleavage products of human gelsolin was studied by metabolic labeling using cells transfected with either full-length gelsolin or its N- or C-terminal cleavage products. The effects of posttranslational N-myristoylation of the C-terminal cleavage product of gelsolin (tGelsolin) on its intracellular targeting and on the antiapoptotic activity were then investigated. The results showed that human tGelsolin was posttranslationally N-myristoylated upon caspase-mediated cleavage. This posttranslationally N-myristoylated tGelsolin was not targeted to mitochondria, but rather was diffusely distributed in the cytoplasm.

Overexpression of N-myristoylated tGelsolin in COS-1 cells using the plasmid tGelsolin-IRE-EGFP significantly inhibited etoposide-induced apoptosis, whereas overexpression of the non-myristoylated tGelsolinG2A mutant did not cause resistance to apoptosis.

These results indicated that posttranslational N-myristoylation of tGelsolin is involved in the anti-apoptotic activity of tGelsolin.

**EXPERIMENTAL PROCEDURES**

**Materials —** Restriction endonucleases, DNA-modifying enzymes, RNase inhibitor, and Taq DNA polymerase were purchased from Takara Shuzo, Kyoto, Japan. RNase was obtained from Boehringer-Mannheim, Germany. [3H]leucine, [3H]myristic acid, and Amplify were from Amersham, UK. The Dye Terminator Cycle Sequencing kit was from Applied Biosystems, USA. Anti-FLAG monoclonal antibody, anti-gelsolin C-terminal fragment monoclonal antibody, anti-heat shock protein 70 (Hsp70) monoclonal antibody and FITC-conjugated anti-mouse IgG antibody were purchased from Sigma, USA. Anti-VDAC polyclonal antibody and ProteoExtract<sup>®</sup> Subcellular Proteome Extraction Kit were purchased from Merck KGaA, Germany. Anti-EGFP antibody was from Santa Cruz Biotechnology, USA. MitoTracker Red CMXRos, Alexa Fluor 594 goat anti-mouse IgG antibody and Hoescht 33342 were obtained from Molecular Probes, Netherlands. Protein G Sepharose was from Pharmacia Biotech, Sweden. Plasmid pCMV-Gelsolin was from OriGene Technologies,
USA. Plasmid pECFP-ER and pRES2-EGFP were obtained from Clontech, USA. Other reagents were purchased from Wako Pure Chemical, Daiichi Pure Chemicals, or Seikagaku Kogyo (Japan) and were of analytical or DNA grade.

**Plasmid construction** — Plasmid pBluescript II SK(+) lacking ApaI and HindII sites was constructed as previously described (17), and designated pB. Plasmid pBG\(_\alpha\)-TNF, which contains a cDNA coding for G\(_\alpha\)-TNF in which N-terminal 10 residues of G\(_\alpha\) protein were linked to the N-terminus of the mature domain of TNF, was constructed as previously described (18). Plasmid pB-FLAG, which contains the sequence for the FLAG epitope at the C-terminus was constructed as described in (7). Plasmid pBtGelsolin-FLAG, which contains a cDNA coding for FLAG-tagged tGelsolin, was constructed by utilizing polymerase chain reaction (PCR). For this procedure, pCMV-Gelsolin (OriGene) served as a template and two oligonucleotides (T-GELSO: 5’-ATATGGATCCATGGGCTGGCGTCC-3’ and GELSO: 5’-ATATGAATTCCGCAAGCGCAGCTTGGCCG-3’) as primers. After digestion with BamHI and EcoRI, the amplified products were subcloned into pB-FLAG at the BamHI and EcoRI sites. Plasmid pBtGelsolinG2A-FLAG was constructed by a method similar to that used to construct pBtGelsolin-FLAG using two oligonucleotides (T-GELSGO2A: 5’-ATATGGATCCATGGGCTGGCGTCC-3’ and GELSO: 5’-ATATGAATTCCGCAAGCGCAGCTTGGCCG-3’) as primers. Plasmid pBGelsolinFLAG, which contains a cDNA coding for long-length gelsolin was constructed by a method similar to that used to construct pBGelsolin-FLAG using two oligonucleotides (N-GELSO: 5’-ATATGGATCCATGGGCTGGCGTCC-3’ and GELSO: 5’-ATATGAATTCCGCAAGCGCAGCTTGGCCG-3’) as primers. Plasmid pBN-Gelsolin-FLAG, which contains a cDNA coding for the FLAG-tagged N-terminal caspase-cleavage product of gelsolin, was constructed by a method similar to that used to construct pBtGelsolin-FLAG using two oligonucleotides (N-GELSO: 5’-ATATGGATCCATGGGCTGGCGTCC-3’ and GELSO: 5’-ATATGAATTCCGCAAGCGCAGCTTGGCCG-3’) as primers. Plasmid pBGelsolin-FLAG was constructed by a method similar to that used to construct plBtGelsolin-FLAG. For this procedure, pBG\(_\alpha\)-TNF served as a template and two oligonucleotides (T3: 5’-AAATTACCCACTAAAGGG-3’ and TNFAC: 5’-GGCGGCGGCAATGGCATCC-3’) as primers. After digestion with BamHI and EcoRI, the amplified products were subcloned into pB-FLAG at the BamHI and EcoRI sites. All the cDNAs in pB vector were subcloned into pcDNA3 and used for transfection assays.

The bicistronic expression vector pRES2-EGFP lacking a BamHI site (pRES2(ΔB)) was constructed by digesting pRES2-EGFP (Clontech) with BamHI, blunt-ending with mung bean nuclease, and ligating with T4 ligase. Plasmid pRES2(ΔB)-FLAG, which contains the sequence for the FLAG epitope at the C-terminus, was constructed by utilizing PCR. For this procedure, pB-FLAG served as a template and two oligonucleotides (T3: 5’-AAATTACCCACTAAAGGG-3’ and C-FLAG-PST: 5’-GGCCTGCGACTTATTGGCT-3’) as primers. After digestion with SacI and PstI, the amplified product was subcloned into pRES2(ΔB) at the SacI and PstI sites. Plasmid pRES2(ΔB)-tGelsolin-FLAG was constructed as follows. The BamHI/EcoRI fragment coding for tGelsolin was excised from pB-tGelsolin-FLAG, and then subcloned into pRES2(ΔB)-FLAG at the BamHI and EcoRI sites. Plasmids pRES2(ΔB)-tGelsolinG2A-FLAG, pRES2(ΔB)-tGelsolin-FLAG and pRES2(ΔB)-tActin-FLAG were constructed by a method similar to that used to construct pRES2(ΔB)-tGelsolin-FLAG.

The DNA sequences of these recombinant cDNAs were confirmed by the dideoxy-nucleotide chain termination method (19).
Transfection of COS-1 cells and determination of N-myristoylated proteins — The simian virus 40-transformed African Green monkey kidney cell line, COS-1, was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS; Gibco BRL). Cells (2x10^6) were plated onto 35-mm diameter dishes 1 day before transfection. pcDNA3- or pIRES2(ΔB) construct (2 µg) containing cDNA coding for mutant gelsolin was used to transfect each plate of COS-1 cells along with 4 µl of LipofectAmine (2 mg/ml; Gibco BRL) in 1 ml of serum-free medium. After incubation for 5 h at 37°C, the cells were refed with serum-containing medium and incubated again at 37°C for 48 h. The cells were then washed twice with 1 ml of serum-free DMEM and incubated for 4 h at 37°C in 1 ml of DMEM with 2% FCS containing [3H]myristic acid (100 µCi/ml). For the treatment with etoposide or staurosporine, 48 h after transfection, the cells were incubated with 200 nM etoposide or 2 µM staurosporine at 37°C for 18 h in 1 ml of DMEM with 5% FCS containing [3H]myristic acid (100 µCi/ml). For the treatment with z-VAD-fmk, the cells were pretreated with 100 µM z-VAD-fmk for 3 h before addition of etoposide or staurosporine. Subsequently, the cells were washed three times with Dulbecco’s phosphate-buffered saline (DPBS) and collected with a cell scraper, and then lysed with 200 µl of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), proteinase inhibitors] on ice for 20 min. After immunoprecipitation with anti-FLAG antibody, the samples were analyzed by SDS-PAGE and fluorography.

Immunoprecipitation — Samples were immunoprecipitated with a specific anti-FLAG or anti-Gelsolin antibody as described in (17).

SDS-PAGE and fluorography — Samples were denatured by boiling for 3 min in SDS-sample buffer followed by analysis by SDS-PAGE on a 12.5% gel. Thereafter, the gel was fixed and soaked in Amplify™ (Amersham) for 20 min. The gel was dried under vacuum and exposed to X-ray film (Kodak) for an appropriate period.

Immunofluorescence analysis and fluorescence microscopy — Immunofluorescence analysis of transfected cells was performed 48 h after transfection. At this time, the maximum level of protein expression was achieved. For immunocytochemistry, cells were washed with DPBS, fixed in 4% paraformaldehyde in DPBS for 15 min, and permeabilized with 0.1% Triton X-100 in DPBS for 10 min at room temperature, followed by washing with 0.1% gelatin in DPBS. The permeabilized cells were incubated with anti-FLAG antibody (1:1000) in DPBS for 1 h at room temperature. After washing with 0.1% gelatin in DPBS, the cells were incubated with
FTTC-conjugated anti-mouse IgG antibody or Alexa Fluor 594 goat anti-mouse IgG antibody for 1 h at room temperature. Mitochondria were identified by incubating the cells with 300 nM MitoTracker red for 30 min before fixation. After washing with 0.1% gelatin in DPBS, the cells were observed under a Zeiss Axiovert fluorescence microscope.

Subcellular fractionation — Subcellular fractionation of COS-1 cells expressing either tGelsolin-FLAG or tGelsolinG2A-FLAG was performed by using ProteoExtract™ Subcellular Proteome Extraction Kit (Merck) according to the manufacturer’s instructions. Briefly, COS-1 cells (2x10⁷) were transfected with 2 µg of pcDNA3tGelsolin-FLAG or pcDNA3tGelsolinG2A-FLAG as described earlier and incubated at 37°C for 48 h. After washing twice with ice cold Wash Buffer, cells were incubated with 0.5 ml of ice cold Extraction Buffer I at 4°C for 10 min, and then the supernatant was collected and used as a cytosolic fraction. Subsequently, cells were incubated with 0.5 ml of ice cold Extraction Buffer II at 4°C for 30 min, and then the supernatant was collected and used as a membrane/organelle fraction. The cells were then incubated with 0.5 ml ice cold Extraction Buffer III at 4°C for 10 min, then the supernatant was collected and used as a nucleic fraction.

Induction and detection of apoptosis — The transfected COS-1 cells were incubated with 200 nM etoposide for 24 h. The cells were stained with 1 µM Hoechst 33342 and observed under a Zeiss Axiovert fluorescence microscope. The cell viability was assessed by examining the nuclear morphology. The numbers of total EGFP-positive cells and EGFP-positive cells showing apoptotic phenotype (Hoechst-positive cells) were counted and the % of apoptosis was calculated. In this case, 4.5 ± 10⁵ cells found in 8 randomly selected area were counted in each sample and data are expressed as mean ± S.D. of three independent experiments.

RESULTS

N-terminus of C-terminal caspase-cleavage product of gelsolin is N-myristoylated — Our previous study showed that the N-terminal nine residues of the C-terminal caspase-cleavage product of human gelsolin (tGelsolin) efficiently direct the protein N-myristoylation (7). To confirm that the full-length tGelsolin is N-myristoylated, cDNAs coding for epitope-tagged gelsolin and tGelsolin were generated and their susceptibility to protein N-myristoylation was evaluated by metabolic labeling in transfected cells. For these analyses, a FLAG-tag was introduced at the C-terminus of these constructs. As shown in Fig. 1A, lane 2, transfection of COS-1 cells with cDNA coding for FLAG-tagged tGelsolin gave rise to a 44 kDa protein band with the expected molecular mass (42 kDa tGelsolin plus 2 kDa linker and FLAG-tag). The 44 kDa protein band was efficiently N-myristoylated, as determined by [3H]myristic acid labeling (Fig. 1A lane 6). When Gly2 of tGelsolin-FLAG was replaced with Ala (tGelsolinG2A-FLAG), no incorporation of [3H]myristic acid into this mutant was observed despite the effective expression of this protein, as shown in Fig. 1A, lanes 3 and 7. Transfection of cDNAs coding for full-length gelsolin (Gelsolin-FLAG) and the N-terminal fragment of gelsolin (N-Gelsolin-FLAG) gave rise to protein bands with the expected molecular mass (88 kDa and 44 kDa, respectively). [3H]-myristic acid incorporation into these proteins was not observed (Fig. 1A, lanes 1, 4, 5 and 8). As shown in Fig. 1B, the efficiency of [3H]myristic acid incorporation ([3H]myristic acid labeling/western blotting) into tGelsolin-FLAG (lanes 3, 6) was comparable to that into tActin-FLAG (lanes 2, 5) and Gαα-TNF-FLAG (lanes 1, 4) having N-myristoylation motif of Gαα protein at its N-terminus (20), indicating that tGelsolin-FLAG is efficiently N-myristoylated.

Induction of apoptosis induces the generation of posttranslationally N-myristoylated tGelsolin — To determine whether the intracellular generation of N-myristoylated tGelsolin is induced by caspase-mediated cleavage of gelsolin during apoptosis, COS-1 cells transfected with FLAG-tagged gelsolin were treated with etoposide or staurosporine, apoptosis-inducing agents, and the generation of N-myristoylated tGelsolin was examined by Western blotting and [3H]myristic acid labeling. As shown in Fig. 2A, lanes 1, 2, 4, 6, 7 and 9, the generation of N-myristoylated tGelsolin was induced by the treatment of cells with 200 nM...
etoposide or 2 µM staurosporine. The induction of the generation of the N-myristoylated tGelsolin was completely inhibited by pretreatment of the cells with 100 µM z-VAD-fmk, a caspase inhibitor, before addition of etoposide or staurosporine (Fig. 2A, lanes 3, 5, 8 and 10). To determine whether the generation of posttranslationally N-myristoylated tGelsolin was observed on endogenous gelsolin during apoptosis, HeLa cells were treated with staurosporine and the generation of N-myristoylated tGelsolin was examined. As shown in Fig. 2B, the generation of N-myristoylated tGelsolin was induced by the treatment of cells with 2 µM staurosporine (lanes 2 and 5) and this induction was completely inhibited by pretreatment of the cells with 100 µM z-VAD-fmk (lanes 3 and 6). These results strongly indicate that the generation of N-myristoylated tGelsolin is induced by caspase-mediated cleavage of gelsolin during apoptosis.

Exogenously expressed tGelsolin does not localize to mitochondria — Previous studies showed that both N-myristoylated tBID and tActin colocalize with mitochondria in an N–myristoylation-dependent manner (6, 7). These results indicate that posttranslational N-myristoylation might function as a mitochondrial-targeting signal. To examine whether the exogenously expressed N-myristoylated tGelsolin colocalizes with mitochondria or not, immunofluorescence staining coupled with staining with MitoTracker, a mitochondria-specific dye, was performed. As previously observed, exogenously expressed tActin colocalized with mitochondria, as shown in Fig. 3 a,b. In contrast, the distribution of exogenously expressed tGelsolin detected by immunofluorescence staining were distinct from that of MitoTracker, indicating that tGelsolin does not colocalize with mitochondria (Fig. 3 c,d). To determine whether the exogenously expressed tGelsolin colocalizes with endoplasmic reticulum or not, tGelsolin-FLAG was coexpressed with ECFP-ER having ER-targeting signal and their localizations were determined by immunofluorescence staining and ECFP-fluorescence, respectively. As shown in Fig. 4A b, ECFP-fluorescence showed reticular distribution around nucleus whereas immunofluorescence staining of tGelsolin-FLAG showed diffuse cytosolic distribution (Fig. 4A a), indicating that tGelsolin does not colocalize with ER. The fact that both tGelsolin-FLAG and tGelsolinG2A-FLAG showed similar diffuse cytosolic distribution indicated that N-myristoylation of tGelsolin is not directly involved in the intracellular targeting of tGelsolin (Fig. 3 c,d,g,h). Similar cytosolic distribution was observed with exogenously expressed Gelsolin-FLAG, as shown in Fig. 3 e,f. To confirm the cytosolic distribution of tGelsolin-FLAG, subcellular fractionation experiment on COS-1 cells expressing either tGelsolin-FLAG or tGelsolinG2A-FLAG were performed. As shown in Fig. 4B, tGelsolin-FLAG and tGelsolinG2A-FLAG were fractionated exclusively in cytosolic fraction as was the case with Hsp70 (heat shock protein 70), a cytosolic marker protein, indicating that both tGelsolin-FLAG and tGelsolinG2A-FLAG were distributed to cytoplasm.

N-myristoylation is required for anti-apoptotic activity of tGelsolin — The anti-apoptotic activity of human gelsolin was shown to reside in the C-terminal caspase-cleavage product, tGelsolin (13). Since posttranslational N-myristoylation occurs on tGelsolin, this modification might be involved in the anti-apoptotic activity of tGelsolin. To determine whether N-myristoylation of tGelsolin is required for its anti-apoptotic activity, the effect of overexpression of N-myristoylated- or non-myristoylated tGelsolin on cellular apoptosis was investigated. For this purpose, the bicistronic expression plasmid ptGelsolin-IRES-EGFP capable of overexpressing human tGelsolin concomitantly with EGFP was constructed. Using this plasmid, the cells overexpressing tGelsolin were easily detected by the presence of EGFP-fluorescence. When EGFP was expressed in COS-1 cells, the number of apoptotic cells with condensed or fragmented nuclei detected by Hoechst staining was about 10 % of the total EGFP-positive cells (Fig. 5 B). When these cells were treated with 200 nM etoposide for 24 h, the number of apoptotic cells increased and 50 % of EGFP-positive cells showed a typical apoptotic phenotype. When human tGelsolin was coexpressed with EGFP by using ptGelsolin-IRES-EGFP, significant inhibition of etoposide-induced apoptosis was observed and only
20% of total EGFP-positive cells showed an apoptotic phenotype. In this case, tGelsolin-expressing cells retained normal cell morphology and intact nuclei, whereas most of the non-transfected cells located around the tGelsolin-expressing cells showed a typical apoptotic phenotype, as shown in Fig. 5 A, c and d. In contrast, when tGelsolinG2A was coexpressed with EGFP, inhibition of etoposide-induced apoptosis was not observed and a similar level of apoptosis (55%) was detected in tGelsolinG2A-expressing cells as compared with EGFP-expressing cells (Fig. 5 A, a, b, g and h). As expected, etoposide-treated tGelsolinG2A-expressing cells showed a typical apoptotic phenotype, as shown in Fig. 5 A, g and h. The expression levels of tGelsolin and GelsolinG2A were similar in the respective transfected cells, as determined by Western blotting analysis (Fig. 5 C), indicating that the difference in the anti-apoptotic activity of these two mutants did not depend on the difference in the expression level of the protein. When intact gelsolin was coexpressed with EGFP, inhibition of etoposide-induced apoptosis was not observed, and more than 40% of EGFP-positive cells showed a typical apoptotic phenotype (Fig. 5 B). In this case, however, the expression level of gelsolin in gelsolin-expressing cells was very low as compared with the levels of the expressed proteins in tGelsolin- or tGelsolinG2A-expressing cells (Fig. 5 C). Therefore, the low level of anti-apoptotic activity of transfected gelsolin might have been due to the low level of its protein expression. We repeated this experiment more than 5 times, and obtained the same result in all cases. Therefore, this result was not due to the experimental error. Further experiments are required to determine the ability of intact gelsolin overexpressed in cells to inhibit apoptosis. 

N-myristoylated tActin could not inhibit apoptosis — As shown in Fig. 5, posttranslational N-myristoylation was found to be critical for the anti-apoptotic activity of tGelsolin. In order to determine whether posttranslational N-myristoylation by itself is sufficient to induce anti-apoptotic activity, the effect of overexpression of N-myristoylated tActin on etoposide-induced apoptosis was investigated. As previously reported, tActin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria (7). In fact, when tActin was coexpressed with EGFP using ptActin-IRES-EGFP, mitochondrial localization of tActin in EGFP-positive cells was observed, as determined by immunofluorescence staining (Fig. 6 A, c and d). In contrast, tGelsolin coexpressed with EGFP was diffusely distributed in the cytoplasm, as previously observed (Fig. 6 A, a and b). When tActin-expressing cells were treated with 200 nM etoposide for 24 h, the number of apoptotic cells increased and about 50% of EGFP-positive cells showed a typical apoptotic phenotype (Fig. 6 B and C). In tGelsolin-expressing cells, significant inhibition of apoptosis was observed and only 20% of EGFP-positive cells showed an apoptotic phenotype (Fig. 6 B and C). The level of the expressed protein in tActin- and tGelsolin-expressing cells was comparable, as determined by Western blotting analysis (Fig. 6 D), indicating that the difference in the anti-apoptotic activity of these two proteins did not depend on the difference in the expression level of the protein. These results clearly indicated that N-myristoylation by itself or mitochondrial targeting by N-myristoylation is not sufficient to induce anti-apoptotic activity.

DISCUSSION

Protein N-myristoylation has long been recognized as a cotranslational protein modification. Recently, it was demonstrated that N-myristoylation can also occur posttranslationally, as in the case of pro-apoptotic protein BID (6). The post-proteolytic N-myristoylation of BID was found to promote its targeting to mitochondria as well as to enhance its pro-apoptotic action in vivo. Since it was shown that many caspase substrates expose an N-terminal Gly upon cleavage, it was suggested that the posttranslational N-myristoylation might be a common modification in the apoptosis pathway (6). Until recently, however, BID was the only protein that had been proved to be posttranslationally N-myristoylated upon caspase-mediated cleavage. Recently, we tried to search for caspase substrates that are posttranslationally N-myristoylated upon caspase-mediated cleavage. For this purpose, the susceptibility of the newly exposed N-terminus of known caspase substrates to protein N-myristoylation

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was evaluated by in vivo metabolic labeling with $[^3]H$myristic acid in transfected cells using a fusion protein in which the query sequence was fused to a model protein. As a result, it was found that the N-terminal sequence of the newly exposed N-terminus of the caspase-cleavage product of cytoskeletal actin efficiently directed protein N-myristoylation. Using full-length human actin cDNA, we demonstrated that the C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and is then targeted to mitochondria (7). In contrast to N-myristoylated tBID, N-myristoylated tActin localized at mitochondria did not induce cellular apoptosis. The physiological role of the mitochondrial localization of tActin remain unclear. During the analysis of posttranslational N-myristoylation of tActin, we also showed that the N-terminal nine residues of the newly exposed N-terminus of the caspase-cleavage product of gelsolin, an actin-regulatory protein, efficiently direct protein N-myristoylation (7). In the present study, the posttranslational N-myristoylation of the caspase-cleavage products of gelsolin was evaluated by in vivo metabolic labeling using cells transfected with either full-length gelsolin or its N- and C-terminal cleavage products. The results showed that tGelsolin is posttranslationally N-myristoylated upon caspase-mediated cleavage during apoptosis. Since both of the posttranslationally N-myristoylated proteins so far discovered (tBID and tActin) localized exclusively to mitochondria, it was speculated that posttranslational N-myristoylation might function as a mitochondrial-targeting signal. In the present study, however, it was revealed that the posttranslationally N-myristoylated tGelsolin did not localize to mitochondria, but rather was diffusely distributed in the cytoplasm (Fig. 3 c). Therefore, it is clear that posttranslational N-myristoylation cannot be a mitochondrial-targeting signal. The intracellular distribution of tGelsolin was not affected by the inhibition of protein N-myristoylation by replacing Gly-2 with Ala (Fig. 3 c, g), indicating that the posttranslational N-myristoylation of tGelsolin is not directly involved in the intracellular localization of this protein.

Gelsolin is an actin-regulatory protein that modulates actin assembly and disassembly, and is believed to regulate cell motility through modulation of the actin network (8, 9). Gelsolin contains six homologous domains (G1-6), each of which has distinct functions (10, 21-25). The N-terminal fragment with G1-3 possesses F-actin-severing activity, and the C-terminal fragment with G4-6 possesses a Ca$^{2+}$-dependent actin monomer binding activity (15, 23, 26). Gelsolin was also recently suggested to be involved in the regulation of apoptosis (10-14, 26, 27). Overexpression of human gelsolin has been shown to inhibit apoptosis through the ability to block the loss of mitochondrial membrane potential and to inhibit caspase activity (13). It was also shown that human gelsolin were cleaved at a single site (D352) of DQTD$^{350}$G within the G3 domain by caspase-3 (15, 16). The anti-apoptotic activity of human gelsolin was shown to reside in the C-terminal caspase-cleavage product, tGelsolin (13). Recently, it was reported that segment 5 in the C-terminal half of gelsolin is an important region for the anti-apoptotic property of human gelsolin (28).

Since posttranslational N-myristoylation occurs on tGelsolin, this modification might be involved in the anti-apoptotic activity of tGelsolin. To study the role of this modification in the anti-apoptotic activity of tGelsolin, we constructed the bicistronic expression plasmid tGelsolin-IRES-EGFP capable of overexpressing tGelsolin concomitantly with EGFP. Using this plasmid, cells overexpressing tGelsolin were easily detected by the presence of EGFP-fluorescence. It was found that overexpression of N-myristoylated tGelsolin in COS-1 cells significantly inhibited etoposide-induced apoptosis, whereas overexpression of non-myristoylated tGelsolinG2A mutant did not inhibit apoptosis. These results clearly indicate that posttranslational N-myristoylation of tGelsolin is directly involved in the anti-apoptotic activity of tGelsolin. The fact that N-myristoylated tActin, which specifically localized to mitochondria in an N-myristoylation-dependent manner, did not show anti-apoptotic activity indicates that N-myristoylation by itself or mitochondrial-targeting is not sufficient to induce anti-apoptotic activity.

The molecular mechanism by which N-myristoylation affects the anti-apoptotic activity of tGelsolin remains to be elucidated. It was reported...
that although most endogenous gelsolin is localized in the cytoplasm (29), a small amount exists in the cytosolic face of the cell membranes, including the mitochondrial membranes (30). This mitochondrial human gelsolin seems to be responsible for the anti-apoptotic activity of this protein. In fact, it was shown that human gelsolin and human tGelsolin could prevent apoptotic mitochondrial changes such as ΔΨ loss and cytochrome c release in isolated mitochondria to a similar extent as human Bcl-xL (12, 13). As shown in Fig. 3 and 4, both N-myristoylated tGelsolin and non-myristoylated tGelsolinG2A mutant were distributed in the cytoplasm, indicating that posttranslational N-myristoylation does not significantly affect the apparent intracellular localization of tGelsolin. Although both intact gelsolin and tGelsolin were shown to have anti-apoptotic activity, it is not clear whether the mode of action of these two molecules is the same. Further studies using purified N-myristoylated tGelsolin and isolated mitochondria will be required to characterize the direct interaction of N-myristoylated tGelsolin with mitochondria. Since gelsolin is an actin-regulatory protein and tGelsolin retains Ca²⁺-dependent actin monomer binding activity (25), it is possible that the interaction of tGelsolin with actin or tActin is involved in the anti-apoptotic activity of tGelsolin.

Another important issue to be clarified is the physiological meaning of the present findings. One complicating fact is that the N-terminal half of the caspase-cleavage product of gelsolin generated upon apoptosis has been reported to have pro-apoptotic activity and induce morphological changes characteristic of apoptosis (15, 31). In fact, it was recently reported that apoptosis induced by interferon-α is mediated by the generation of the N-terminal half of gelsolin through caspase-3 activation (28). Further investigation will be required to clarify the roles of the N- and C-terminal caspase-cleavage products of gelsolin in the regulation of apoptosis.

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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** N-terminus of C-terminal caspase-cleavage product of gelsolin is N-myristoylated.

A. Gelsolin-, tGelsolin-, tGelsolinG2A- and N-Gelsolin-FLAG were expressed in COS-1 cells and the cells were labeled with [3H]myristic acid.

Left panel: Total cell lysates were analyzed by Western blotting using anti-FLAG antibody.

Right panel: Following immunoprecipitation with anti-FLAG antibody, the labeled proteins were analyzed by SDS-PAGE and fluorography.

B. G,α-TNF-, tActin- and tGelsolin-FLAG were expressed in COS-1 cells and the cells were labeled with [3H]myristic acid.

Left panel: Total cell lysates were analyzed by Western blotting using anti-FLAG antibody.

Right panel: Following immunoprecipitation with anti-FLAG antibody, the labeled proteins were analyzed by SDS-PAGE and fluorography.

**Fig. 2.** Induction of apoptosis induces the generation of N-myristoylated tGelsolin.
A. COS-1 cells transfected with cDNA coding for Gelsolin-FLAG were incubated with 200 nM etoposide or 2 μM staurosporine at 37°C for 18 h in 1 ml of DMEM with 5% FCS containing [3H]myristic acid (100 μCi/ml). For treatment with z- VAD-fmk, the cells were pretreated with 100 μM z-VAD-fmk for 3 h before the addition of etoposide or staurosporine.

Left panel: Total cell lysates were analyzed by Western blotting using anti-FLAG antibody.
Right panel: Following immunoprecipitation with anti-FLAG antibody, the labeled proteins were analyzed by SDS-PAGE and fluorography.

B. HeLa cells were incubated with 2 μM staurosporine at 37°C for 10 h in 1 ml of DMEM with 5% FCS containing 200 μCi [3H] myristic acid. After incubation, the cells were harvested and lysed with RIPA buffer and gelsolin and tGelsolin were immunoprecipitated with anti-gelsolin C-terminal fragment antibody. For the treatment with z-VAD-fmk, the cells were pretreated with 100 μM z-VAD-fmk for 3 h before addition of staurosporine.

Left panel: The samples were analyzed by Western blotting using anti-gelsolin C-terminal antibody.
Right panel: The samples were analyzed by SDS-PAGE and fluorography.

Fig. 3. Exogenously expressed tGelsolin does not localize to mitochondria.
tActin-, tGelsolin-, Gelsolin- and tGelsolinG2A-FLAG were expressed in COS-1 cells.
a,c,e,g: Subcellular localization of tActin- (panel a), tGelsolin- (panel c), Gelsolin- (panel e) and tGelsolinG2A-FLAG (panel g) expressed in COS-1 cells was determined by immunofluorescence staining using anti-FLAG antibody.
b,d,f,h: Mitochondria in the same cells as in panels a,c,e,g, respectively were identified by MitoTracker staining.

Fig. 4. Analysis of subcellular localization of tGelsolin.
A. tGelsolin-FLAG was coexpressed with ECFP-ER having ER-targeting signal and their localizations were determined by immunofluorescence staining (a) and ECFP-fluorescence (b), respectively.
B. tGelsolin-FLAG and tGelsolinG2A-FLAG were expressed in COS-1 cells and subcellular fractionation experiments were performed. Hsp70 (heat shock protein 70) was used as cytosolic marker protein and VDAC (voltage-dependent anion channel) and PDI (protein disulfide isomerase) were used as membrane/organelle marker proteins.
C: cytosolic fraction;  M: membrane/organelle fraction;  N: nucleic fraction.

Fig. 5. N-myristoylation is required for anti-apoptotic activity of tGelsolin
tGelsolin-, tGelsolinG2A- and Gelsolin-FLAG were co-expressed with EGFP in COS-1 cells. COS-1 cells expressing FGFP were used as a control. After incubation with 200 nM etoposide at 37°C for 24 h, the cells were stained with 1 μM Hoechst 33342, and then EGFP-fluorescence and nuclear morphology were analyzed by immunofluorescence microscopy.

A. a,c,e,g: EGFP-fluorescence of COS-1 cells expressing EGFP (a), tGelsolin-FLAG/EGFP (c), Gelsolin-FLAG/EGFP (e) and tGelsolinG2A-FLAG/EGFP (g).
b,d,f,h: Nuclear morphology in the same cells as in (a),(c),(e),(g), respectively, analyzed by Hoechst 33342 staining.

B. Susceptibility of COS-1 cells expressing EGFP, tGelsolin-FLAG/EGFP, Gelsolin-FLAG/EGFP and tGelsolinG2A-FLAG/EGFP to etoposide-induced apoptosis. Numbers of total EGFP-positive cells and
EGFP-positive cells showing an apoptotic phenotype (Hoechst-positive cells) were counted and the % of apoptosis was calculated. Data are expressed as mean ± S.D. of three independent experiments.

C. Total cell lysates of the cells used in A and B were analyzed by Western blotting using anti-FLAG- and anti-EGFP antibody.

Fig. 6. N-myristoylated tActin did not inhibit apoptosis.

*tGelsolin-, and tActin-FLAG were co-expressed with EGFP in COS-1 cells. COS-1 cells expressing FGFP were used as a control.

A. a,c: EGFP-fluorescence of COS-1 cells expressing tGelsolin-FLAG/EGFP (a) and tActin-FLAG/EGFP (c).

b,d: Subcellular localization of tGelsolin-FLAG (b) and tActin-FLAG (d) in the same cells as in (a) and (c) was determined by immunofluorescence staining using anti-FLAG antibody.

B. After treatment with 200 nM etoposide at 37°C for 24 h, the cells were stained with 1 µM Hoechst 33342, and then EGFP-fluorescence and nuclear morphology were analyzed by immunofluorescence microscopy.

a,c: EGFP-fluorescence of COS-1 cells expressing tGelsolin-FLAG/EGFP (a) and tActin-FLAG/EGFP (c).

b,d: Nuclear morphology of the same cells as in (a) and (c) analyzed by Hoechst 33342 staining.

C. Susceptibility of COS-1 cells expressing EGFP, tGelsolin-FLAG/EGFP and tActin-FLAG/EGFP to etoposide-induced apoptosis. Numbers of total EGFP-positive cells and EGFP-positive cells showing an apoptotic phenotype (Hoechst positive cells) were counted and the % of apoptosis was calculated. Data are expressed as mean ± S.D. of three independent experiments.

D. Total cell lysates of the cells used in A, B and C were analyzed by Western blotting using anti-FLAG- and anti-EGFP antibody.

FOOTNOTES

1The abbreviations used are: tGelsolin, truncated gelsolin; tActin, truncated actin; TNF, tumor necrosis factor; NMT, N-myristoyltransferase; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; DPBS, Dulbecco’s phosphate buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis
Fig. 1

Western blotting

[**A**]

- Gelsolin-FLAG
- tGelsolin-FLAG
- tGelsolinG2A-FLAG
- N-Gelsolin-FLAG
- Gi1-TNF-FLAG
- tActin-FLAG

Western blotting

[**B**]

- Gαs-TNF-
- tActin-
- tGelsolin-

[3H] myristic acid labeling

[3H] myristic acid labeling
Fig. 2

[A] Western blotting with Etoposide and STS treatments, followed by [3H] myristic acid labeling in the presence or absence of z-VAD.

[B] Western blotting with STS treatments, followed by [3H] myristic acid labeling in the presence or absence of z-VAD.
Fig. 4
Fig. 5

[A] EGFP

[B] Apoptosis (%)

[C] Westernblotting

Gelsolin

tGelsolin

EGFP

Hoechst
**[Fig. 6]**

- **[A]**
  - EGFP
  - FLAG
  - tGelsolin-
  - tActin-

- **[B]**
  - EGFP
  - Hoechst
  - tGelsolin-
  - tActin-

- **[C]**
  - Bar graph showing Apoptosis (%) in response to Etoposide treatment.
  - EGFP
  - tAct
  - tGel

- **[D]**
  - Western blot analysis with bands for EGFP, tGelsolin, tActin, and FLAG.
Posttranslational N-myristoylation is required for the anti-apoptotic activity of human tGelsolin, the C-terminal caspase-cleavage product of human gelsolin

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