In vitro Investigation of Actin Cleavage by Apoptotic Proteases, Granzyme B and Caspase-3

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Abstract One of the fundamental changes during apoptosis is the abnormality of cytoskeleton proteins, which determines some morphological features of apoptotic cells. To understand the role of apoptotic proteases, granzyme B and caspase-3, in the cleavage of the cytoplasmic form of actin, an alternative cell free system based on adult monkey brain tissue was used to reproduce the downstream part of apoptotic program, initiated by the addition of granzyme B. Through extensive Western blot analyses, it was showed that β-actin was cleaved to 41 and 15 ku fragments in the granzyme B-treated brain extract after a 12-hour incubation. The production of these two fragments was further found to be granzyme B dependent. Neither endogenous caspase-3 activated by granzyme B nor its recombinant active form was capable of processing the actin in the brain extract, although the enzyme cleaved the actin purified from rabbit skeleton muscle to produce the 15 ku fragment. The results suggest that endogenous β-actin is resistant to apoptotic proteases, especially to caspase-3, either because of conformational constrains between actin and these proteases or due to the presence of other factors that prevent degradation.

Key words apoptosis, actin, granzyme B, caspase-3, proteolysis, Western blotting

Apoptosis is a programmed form of cell death that is now widely recognized as being of critical importance in health and disease. Although highly characteristic morphological changes such as chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing and apoptotic body formation have been used as the basis of the classical definition of apoptosis for more than 30 years[1], it is only recently that insights have been gained into the biochemical mechanisms which underlie these stereotypical ultrastructural features. Accumulating evidence to date emphasizes the roles of proteases in the cell death program and much attention has focused on the interleukin-1β (IL-1β)-converting enzyme (ICE)-like proteases known as caspases, due to the enormous stride made by Horvitz and his colleagues in understanding programmed cell death in the nematode *Caenorhabditis elegans*[2]. During apoptosis, caspase family proteases act in different phases as transmitters or executioners of death signals[3]. However, there is other substantial evidence supporting the involvement of non-caspase proteases in this form of cell death. Amongst them, granzyme B is the only one ever described to share a primary specificity for Asp with caspases and is postulated to cleave intracellular proteins directly and indirectly via activating caspases[4,5].

In the last few years, intracellular targets of the apoptosis-related proteases have been considered to be the final checkpoint in death signal pathways as their cleavage results in most, if not all, of the morphological and functional changes in apoptotic cells. For example, cleavage of nuclear lamins by the proteases induces apoptotic nuclear condensation[6]. Likewise, cleavage of ICAD/DFF45 by caspase-3 leads to the activation of caspase-activated DNase (CAD), an endonuclease that is responsible for apoptotic DNA fragmentation[7]. Comparable scenarios can be envisioned for other polypeptides that are cleaved by specific proteases during the cell death. Since significant information about the role of proteolysis in apoptosis will be gained by identification of the distal substrates on which caspases or other proteases act as pro-apoptotic components, identification of these substrates therefore remains an important challenge.

In view of the dramatic rearrangement in subcellular structures, which is followed by morphological changes and cellular fragmentation, several previous studies focused on cytoskeletal components and their related proteins as potential targets of proteolysis. Their results demonstrated that some of these proteins, e. g. actin, gelsolin, and fodrin, are proteolytically cleaved during apoptosis and caspase-3 is involved in these processes[8~10]. In addition, Mashima *et al.*[11] observed a shrunken morphology in 293T cells transfected with actin-fragment expression vector, suggesting a positive role that the cleaved actin may play in morphological apoptosis. However, conflicting data still exist as to whether actin is subject to cleavage during apoptosis. For example, Song *et al.*[12] did not observe the cleavage of actin in several types of human cells undergoing typical apoptosis induced by various stimuli. Similar results were obtained in some tumor cell lines derived from rodents[13]. The reason for this

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discrepancy remains unclear. It is possible that cytoplasmic compartment may exist between actin and its protease candidate(s) that to some extent determines the status of actin filament during apoptosis in a cell type- or/and stimulus-specific manner. Considering the remarkable alteration in actin aggregation in apoptotic cells and the positive detection of actin cleavage in several neuropathological conditions in which apoptosis plays a role, we assumed that the cleavage of endogenous actin may occur in an alternative cell-free system which was previously established to detect potential cleavage targets of caspase-3 and granzyme B in cytoplasm[14]. In this study we found that cytoplasmic actin in the brain extract was not susceptible to caspase-3 activity but it could be processed by granzyme B. It suggests that while actin contains the cleavage site for caspase-3 and its purified form can be chopped in an *in vitro* assay, certain unknown component(s) in this cytosol-like complex may be responsible for the resistance of actin to degradation.

1 Materials and methods

1.1 Chemical reagents

Purified granzyme B was purchased from Calbiochem (San Diago, CA) and rabbit skeleton muscle actin from Sigma Chemical Co. (St Louis, MO). Recombinant human active caspase-3 protein (rCaspase-3), Z-VAD-FMK and Ac-DEVD-CHO were products of Chemicon (Temecula, CA). Anti-PARP monoclonal antibody (clone C2-10) was obtained from PharMingen (San Diego, CA). Anti-glutamine synthetase (Gl Syn, C-20), anti-glial fibrillary acidic protein (GFAP, C-19) and anti-neuronal nitric oxide synthase (nNOS, K-20) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used to probe actin included anti-β-actin monoclonal antibody (clone AC-15) and anti-actin polyclonal antibody (I-19) and were purchased from Sigma and from Santa Cruz Biotechnology, respectively. Anti-mouse IgG secondary antibody conjugated with peroxidase was obtained from Transduction Laboratories (Lexington, KY), whereas anti-rabbit and anti-goat antibodies were from Santa Cruz Biotechnology. Prestained SDS-PAGE protein molecular mass standard was the product of Bio-Rad (Hercules, CA) and an enhanced chemiluminescence detection system, ECL-plus kit, was purchased from Amersham-Pharmacia (Buckinghamshire, England). All other chemicals were of analytical grade and were from Bio-Rad or Sigma.

1.2 Brain tissue

A 4-year-old female rhesus monkey (*Macaca mulatta*) was supplied by the Laboratory Animal Center in the Academy of Military Medical Sciences, Beijing, China. All efforts were made to minimize the animal's sufferings in accordance with the Guide for the Care and Use of Laboratory Animals. The animal was first anesthetized with ketamine hydrochloride (10 mg/kg, i. m.) and then was sacrificed by exsanguination. Brain tissue blocks dissected from the motor cortex were immediately frozen by immersion in liquid nitrogen and then stored at −80℃ until use.

1.3 Preparation of brain tissue extract and actin solution

Each brain tissue block (100 mg wet weight) was homogenized with a Dounce homogenizer in 0.5 ml of 50 mmol/L HEPES buffer (pH 7.4) containing 0.1 mol/L NaCl, 0.1% CHAPS, 10% sucrose, 1 mmol/L EDTA, 10 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 2 mg/L each of leupeptin, pepstatin A, and aprotinin. The homogenate was centrifuged for 30 min at 20 000 *g* and 4℃. The supernatant fraction obtained was either used immediately or stored at −80℃. Protein concentration was determined by Bicinchoninic acid assay (BCA kit, Pierce).

Purified skeleton muscle actin was insoluble in water but easily dissolved in 2 mmol/L Tris-HCl buffer (pH 8.0) containing 0.2 mmol/L $CaCl_2$, 0.2 mmol/L ATP and 0.2 mmol/L dithiothreitol in the concentration of 2 g/L. Aliquots of 4 μg actin were incubated with varying concentrations of granzyme B or recombinant caspase-3, followed by Western blot analysis as described below.

1.4 Cleavage reaction and Western blot analysis

Monkey brain extract containing 100 μg total protein was incubated at 37℃ in the presence or absence of purified granzyme B (25 ng), in a final volume of 30 μl. For prolonged incubation period, dithiothreitol and protease inhibitors were added to the reaction mixture one time every 6 h. At each time point, aliquots were removed and then submitted to either 7.5% (for PARP, nNOS) or 12% SDS-PAGE (for GFAP, Gl Syn and actin). Transfer and

Table 1 Antibodies used in Western blot analysis

Antigens	Epitopes	Dilution of antibodies	*M*(antigens) /ku[1)]
Caspase-3	full length	1∶2 000	32
β-actin	N-terminus	1∶5 000	42
Actin	C-terminus	1∶1 000	42
PARP	216 ~ 375 aa	1∶1 000	116
Gl Syn	C-terminus	1∶1 000	45
nNOS	N-terminus	1∶2 000	155
GFAP	C-terminus	1∶1 500	40 ~ 47

[1)] Apparent molecular mass.

immunodetection were performed as described previously[14]. Briefly, blots were probed with different dilutions of primary antibodies listed in Table 1. After a thorough wash for 30 min with several changes of PBST, blots were incubated for 1 h with peroxidase-conjugated secondary antibodies in blocking solution, followed by detection with ECL-plus kit and exposure to ECL Hyperfilm (Amersham).

2 Results

2.1 Selective proteolysis of proteins in granzyme B-treated brain extract

On the basis of our previous work in which exogenous granzyme B efficiently cleaved and activated caspase-3 in adult monkey brain extract, this granzyme B-treated brain extract can be used as a cell-free system instead of that from carcinoma cell lines to investigate potential targets of caspase-3 and granzyme B in the matured primate tissue[14]. In this reaction mixture, PARP, an enzyme involved in DNA repair, was definitely cleaved to produce an 85 ku fragment which is known specific to caspase-3 activity (Figure 1)[15]. To ascertain if other proteins in the extract may degrade in the presence of granzyme B and active forms of caspase-3, we performed immunoblot analysis for nNOS, glutamate synthetase, and GFAP. The results showed that these cytoplasmic proteins remain unchanged after a 12-hour incubation in the presence of granzyme B. It suggests that apoptotic proteolysis may be limited to the intracellular proteins that play critical roles in cell homeostasis and not to those involved in tissue-specific structure or function.

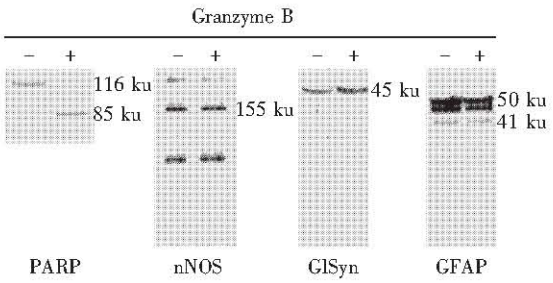

Fig. 1 Selective proteolysis of intracellular proteins in granzyme B -treated monkey brain extracts

Brain extracts (100 μg protein aliquots) were incubated in the presence or absence of granzyme B (25 ng) at 37℃ for 1 h (PARP) or 12 h (other proteins), and then analyzed through Western blotting as described in **Materials and methods.**

2.2 Cleavage of β-actin in granzyme B-treated brain extract

To determine whether any of relatively abundant cellular proteins such as actin serves as the substrate of apoptosis-related proteases, we treated the brain extract with granzyme B and analyzed the products by SDS-PAGE followed by immunodetection. As shown in Figure 2a, the full-length β-actin is a 42 ku protein and it remained intact for at least 24 h when incubated without granzyme B. However, in the presence of granzyme B, the extract displayed a significant reduction in the 42 ku protein after an incubation for 12 h. Meanwhile, smaller cleavage products were not detected by using the anti-β-actin monoclonal antibody throughout the incubation period. Lost of the full-length actin is probably due to the cleavage that occurred near the N-terminal end of β-actin, where the recognition motif of clone AC-15 is located. The cleavage at this site was then confirmed by using a polyclonal antibody against the C-terminal end of actin (Figure 2b). With this anti-C -terminal antibody, the 42 ku full length actin was found to decrease in abundance after a 12-hour incubation and concurrently, a band immediately beneath it (41 ku)

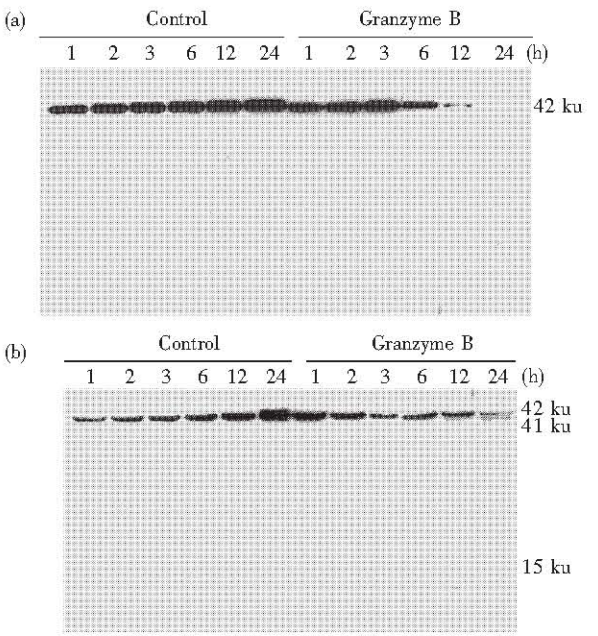

Fig. 2 Cleavage of β-actin in brain extracts treated with granzyme B

Brain extracts (100 μg protein in each tube) were incubated for indicated time periods with or without granzyme B (25 ng) followed by Western blotting as described in **Materials and methods.** Blots were probed using a monoclonal antibody against β-actin (a) or a polyclonal one against the C-terminal end of actin (b).

appeared and its intensity increased with time. In addition, a weakly stained fragment approximately of 15 ku was also observed in the granzyme B-treated samples at the end of incubation period. The production of both 41 ku and 15 ku fragments was resistant to the caspase-3 selective inhibitor, Ac-DEVD-CHO (Figure 3, lane *2 ~ 6*), and the pan-caspase inhibitor, Z-VAD-FMK (data not shown). In agreement with this result, the addition of recombinant

active caspase-3 to the extracts instead of granzyme B did not induce the cleavage of endogenous β-actin (Figure 3, lane *7* and *8*).

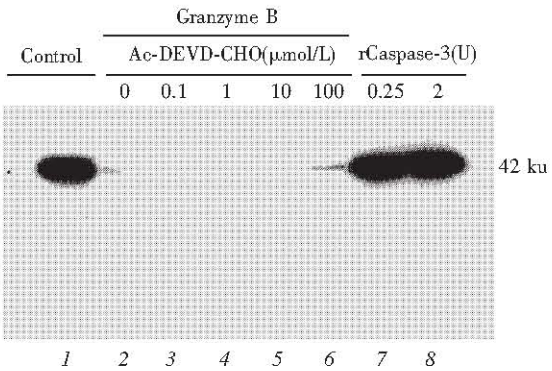

Fig. 3 Cleavage of β-actin in the brain extracts treated with granzyme B, but not in those treated with recombinant active caspase-3 (rCaspase-3)

Brain sample aliquots, 30 μg protein for each, were incubated at 37℃ for 24 h with or without exposure to granzyme B (10 ng), Ac-DEVD-CHO and rCaspase-3 in a final volume of 20 μl. Proteins were separated and detected through Western blotting with the monoclonal antibody against β-actin.

2.3 Determination of purified actin as the substrate of granzyme B and caspase-3

Actin is a highly conserved family of protein in mammals. Within its three subtypes, only two regions (residues 2 ~ 17 and 259 ~ 298) exhibit a high number of replacement in amino acid sequences[16]. Although one of two ICE (caspase-1)-cleavage sites identified by a previous work is located in the first region[17], the amino-acid sequences around the second cleavage site are identical for α- and β-actin. In order to demonstrate whether the alterations in the migration of actin in brain extracts result from the direct cleavage by either granzyme B or caspase-3, the commercially available α - actin instead of endogenous β - actin was incubated with purified granzyme B or recombinant caspase-3, and the products were revealed through Western blot analysis (Figure 4). In the presence of high concentrations of granzyme B, α-actin was cleaved which was demonstrated by the appearance of a 15 ku fragment (lane *3* and *4*). The cleavage was also observed when α-actin was exposed to active caspase-3, but with a greater amount of 15 ku fragment produced (lane *7* and *8*). However, the 41 ku fragment, which was found in the granzyme B-treated brain extracts, was not detected during the incubation of α-actin with granzyme B, nor was it detected with recombinant caspase-3.

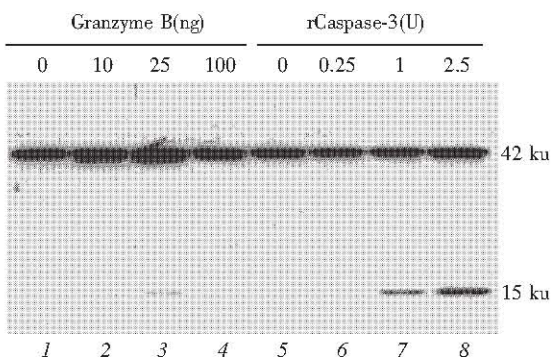

Fig. 4 Cleavage of purified α-actin by granzyme B or by recombinant caspase-3 (rCaspase-3)

α-actin purified from rabbit skeleton muscle (4 μg) was incubated for 2 h with the indicated amounts of granzyme B or rCaspase-3 in a final volume of 20 μl. Proteins were separated and detected through Western blotting using the polyclonal antibody against C-terminal end of actin.

3 Discussion

Proteolysis is the central biochemical process in apoptosis regardless of cell types or kinds of the stimuli. Since the majority of proteins remain intact in cells undergoing apoptosis as determined by high-resolution two-dimensional gel electrophoresis[18], the cleavage by apoptotic proteases is highly selective at earlier stages of apoptosis[19, 20]. Studies in recent years have pointed out that much of the proteolysis is directed by caspases. Other proteases, such as granzymes and calpain, are also involved in the apoptotic signaling process, but in a cell type and/or stimulus-specific manner[21]. Unlike proteolytic mechanisms that help degrade a particular protein and facilitate its timely removal from a cell, cellular proteins targeted by caspases undergo restricted proteolysis that activates a "caspase cascade" and accelerates cell structure collapse.

The linkage between the characteristic morphological changes in apoptotic cells and the specific degradation of actin was first established by Kayalar *et al.* [17] who showed that actin in PC12 cells was degraded at 24 h after serum withdrawal. The cleaved actin has a reduced capacity to inhibit DNase Ⅰ, an enzyme implicated in DNA fragmentation during apoptosis[22]. In addition, the cleavage products are diminished in their ability to polymerize. On the basis of their findings, Kayalar proposed a model in which actin normally inhibits DNase Ⅰ, but on the cleavage of actin by a caspase-like protease, the endonuclease is free to enter the nucleus and fragmentizes DNA. The degradation of actin, on the other hand, would disrupt the cytoskeleton and contribute to the morphological changes of apoptotic cells. This hypothesis is attractive, but it depends largely on the universality of actin cleavage in various types of apoptotic cells. Results of recent studies on the fate of actin during apoptosis are controversial. Mashima and his colleagues[23, 24] showed that actin was cleaved when it was incubated in VP-16-treated U937 cell lysate and the cleavage also occurred *in vivo* in the

same type of cells undergoing apoptosis. Furthermore, they demonstrated that CPP-32/apopain (caspase-3) is responsible for this effect as its selective inhibitor, Z-EVD-CH_2-DCB, efficiently abolished the cleavage and the apoptosis of VP-16-treated U937 cells as well. Although actin degradation was also observed in either physiological or pathological conditions such as neutrophil turnover[25], brain damage[26] and Alzheimer's disease brain[27], no evidence was found to support a direct role of caspase-3 in each of these processes.

Our present work shows that cytoplasmic actin remains unchanged in the monkey brain extract incubated with recombinant active caspase-3. On the contrary, purified α-actin was cleaved by caspease-3 to produce the 15 ku fragment in a buffer condition unfavorable to actin polymerization[28]. With the identity of the tetrapeptide (P4 ~ P1) N-terminal to the cleavage site, Asp^{244} (P1)-Gly^{245}, for these two actin isoforms regardless of their origin, the failure in detecting the degradation of endogenous actin (largely polymerized) indicates that depolymerized actin (G-actin) may be susceptible to the cleavage after the residue Asp^{244}. This partially explains why purified actin was inevitably degraded when incubated with recombinant caspase-3 or apoptotic lysate, whereas endogenous actin appeared resistant to the cleavage either *in vitro* (our results) or *in vivo*[12,13]. In contrast to caspase-3, granzyme B directly cleaved β-actin at two sites (Asp^{11}-Asn^{12} and Asp^{244}-Gly^{245}) to generate several products among which the 41 ku and 15 ku fragments can be detected on the blot probed with anti-C-terminal antibody (Figure 5). In addition, Asp^{11}-Asn^{12} is the major cleavage site for granzyme B in view of the time course of proteolysis and the relative yields of these two products. However, granzyme B was unable to cleave α-actin at the N-terminal region. A previous study revealed that the substrate specificity of granzyme B is highly dependent on a six amino acid sequence that spans over the site of cleavage in its presumed protein substrate[29]. The optimal substrate sequence has been determined to be IEXD ↓ XG with the cleavage at the D ↓ X peptide bond. X represents that granzyme B can accept a broad range of amino acids at the P2 and P1′ positions, although proline and uncharged amino acids are preferred, respectively. The sequences at the potential cleavage sites near N-terminal end of actin are LVVD ↓ NG for β-actin and LVCD ↓ NG for α-actin. Although neither of them is optimal for granzyme B to cleave, they contain more than half of the defined specificity determinants of this protease. The preference of granzyme B to β-actin at its N-terminal cleavage site may be dictated by the difference in residues at the P2 position (Val *vs.* Cys), though direct evidence remains to be obtained through site mutagenesis.

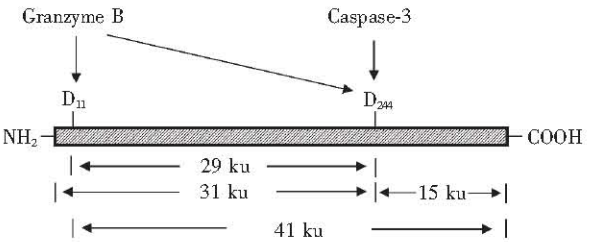

Fig. 5 Schematic view of putative cleavage site of β-actin mediated by granzyme B and caspase-3

β-actin is a 42 ku protein comprising 375 amino acid residues. The Cleavages at Asp^{11} and Asp^{244} yield several fragments. Granzyme B cleaves β-actin at both sites, whereas caspase-3 acts mainly at the latter.

Taken together, actin shows low susceptibility to cleavage, possibly due to its lack of the sequences preferred by caspase-3 and granzyme B, or to its special conformation (e. g. polymerization state) that may impede the access of proteases. We could not exclude the possibility of other factors that may participate in the regulation of actin cleavage or of other proteases instead of caspase-3 and granzyme B that are responsible for actin proteolysis in certain conditions of apoptosis. And the fact that granzyme B cleaved actin at two sites supports the view that granzyme B may act with a different cleavage specificity from that of caspase-3. This situation has also been observed in the cleavage of many other substrates such as PARP, DNA-PK_{cs} and ICAD/DFF45. The identification of their substrate spectrum will further expand our knowledge on the role of selective proteolysis in apoptotic cell death.

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颗粒酶 B 和胱天蛋白酶-3 对肌动蛋白水解作用的体外研究

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摘要 已知凋亡过程的基本变化之一是细胞骨架的异常，后者在某种程度上决定凋亡细胞的形态学特征. 为揭示凋亡相关蛋白酶——颗粒酶 B 和胱天蛋白酶-3 对胞浆型肌动蛋白的水解作用，采用成年猕猴脑组织粗提物作为无细胞体系，以外源性颗粒酶 B 触发凋亡途径的终末反应. 经一系列免疫印迹分析发现：孵育 12 h 方见 β-肌动蛋白被剪切，产生 41 ku 和 15 ku 水解片段，并证明该水解反应为颗粒酶 B 依赖；颗粒酶 B 活化的内源性胱天蛋白酶-3 和重组胱天蛋白酶-3 均不能水解脑提取物中的 β-肌动蛋白，尽管胱天蛋白酶-3 可作用于纯化的肌动蛋白，产生 15 ku 片段. 以上结果提示，内源性 β-肌动蛋白对凋亡相关蛋白酶，尤其胱天蛋白酶-3 不敏感，这可能与该蛋白质的空间结构特征或脑组织中存在的某种蛋白酶抑制因子有关.

关键词 凋亡，肌动蛋白，颗粒酶 B，胱天蛋白酶-3，蛋白质水解，免疫印迹分析

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