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Protease Activation in Apoptosis Induced by MAL

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The proteolytic caspase cascade plays a central role in the signaling and execution steps of apoptosis. This study investigated the activation of different caspases in apoptosis induced by MAL (a folding variant of human α-lactalbumin) isolated from human milk. Our results show that the caspase-3-like enzymes, and to a lesser extent the caspase-6-like enzymes, were activated in Jurkat and A549 cells exposed to MAL. Activated caspases subsequently cleaved several protein substrates, including PARP, lamin B, and α-fodrin. A broad-range caspase inhibitor, zVAD-fmk, blocked the caspase activation, the cleavage of proteins, and DNA fragmentation, indicating an important role for caspase activation in MAL-induced apoptosis. Since an antagonistic anti-CD95 receptor antibody, ZB4, did not influence the MAL-induced killing, we conclude that this process does not involve the CD95-mediated pathway. While MAL did not directly activate caspases in the cytosol, it colocalized with mitochondria and induced the release of cytochrome *c*. Thus, these results demonstrate that caspases are activated and involved in apoptosis induced by MAL and that direct interaction of MAL with mitochondria leads to the release of cytochrome *c*, suggesting that this release is an important step in the initiation and/or amplification of the caspase cascade in these cells.

Key Words: **proteases; cytochrome *c;* apoptosis; milk protein complex.**

INTRODUCTION

Apoptosis is a conserved mechanism of cell death involved in embryogenesis, normal cell turnover, and different pathological disorders. A unique family of proteases, the caspases (cysteine-containing aspartate-specific proteases), appears to play a central role in initiation and execution of the apoptotic process. The caspases are constitutively expressed in all cell types as zymogens, or procaspases, that have to be proteolytically cleaved to become active enzymes. At present, 14 members of the human caspase family have been cloned, and they can be divided into three distinct groups according to the tetrapeptide sequence at which they preferentially cleave specific substrates. The caspase-1-like enzymes (caspase-1, -4, and -5) prefer the cleavage site WEHD [1] and have mainly been implicated in the activation of proinflammatory cytokines in immune responses [2]. The caspase-3-like enzymes (caspase-2, -3, and -7) and caspase-6-like enzymes (caspase-6, -8, and -9) have preferences for DEXD and (I/L/V)EXD, respectively, and have all been implicated in the initiation and/or execution of apoptosis [1, 2]. The cleavage sites for caspase-10, -11, -12, -13, and -14 are not yet clear.

The mechanisms of caspase activation are still not fully understood. However, many apoptosis-inducing agents trigger the release of mitochondrial constituents into the cytoplasm, and cell-free assays of caspase activation have revealed that one of the mitochondrial proteins, cytochrome *c*, is a very powerful inducer of procaspase-3 activation [3, 4]. In the cytosol, cytochrome *c* binds to the apoptotic protease-activating factor-1, which interacts with dATP and procaspase-9 to form the "apoptosome" complex [5, 6]. Subsequent activation of procaspase-9 initiates a caspase cascade involving the downstream executioners, caspase-3, -6, and -7. Although it is still unknown how general this mechanism is in activation of the apoptotic proteolytic cascade, there is rapidly accumulating evidence for its importance.

We recently isolated a protein fraction from human milk containing a folding variant of α-lactalbumin (MAL),[3] that induces apoptosis in tumor cells and im-

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[3] Abbreviations used: Ac-DEVD-amc, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ac-IETD-amc, Ac-Ile-Glu-Thr-Asp-amino-4-methylcoumarin; ALA, α-lactalbumin; Chaps, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonic acid; DEVD-cmk, Asp-Glu-Val-Asp-chloromethyl ketone; DTT, dithiothreitol; MAL, multimeric α-lactalbumin; mAb, monoclonal antibody; Mops, 3-[*N*-morpholino]propanesulfonic acid; PARP, poly(ADP-ribose)-polymerase; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PMSF, α-phenylmethylsulfonyl fluoride; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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mature cells [7]. The activity of MAL was shown to depend on the three-dimensional structure of the protein, which was different from the native form [8]. When added to cells, MAL binds to the plasma membrane and is internalized and, subsequently, translocated from the cytoplasm into the nucleus [9]. The extent of accumulation of MAL in the nucleus varies between cell types and is related to the sensitivity of the particular cell type to MAL-induced killing. However, it is still unknown precisely how MAL enters target cells and how it induces apoptosis. This study examines caspase activation in Jurkat (human T-lymphoblastoid leukemia) and A549 (human lung carcinoma) cells undergoing apoptosis after treatment with MAL. Our results show that the caspase-3-like and caspase-6-like enzymes were activated and cleaved different protein substrates. While MAL did not directly activate caspases in the cytosol, it interacted with mitochondria and induced cytochrome *c* release, suggesting that this release is an important step in the initiation and/or amplification of the caspase cascade in these cells.

MATERIALS AND METHODS

Reagents and antibodies. Anti-p17 pAbs were a kind gift from Dr. D. W. Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada), anti-PARP mAbs were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and anti-lamin B mAbs were obtained from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, UK). Anti-α-fodrin mAbs were from Affiniti Research Products Ltd. (Exeter, UK), anti-cytochrome *c* mAbs were a gift from Dr. R. Jemmerson (University of Minnesota Medical School, Minneapolis), and anti-CD95 mAbs (clone CH11 and ZB4) were from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). Secondary goat anti-rabbit and goat anti-mouse Abs were purchased from Pierce (Rockford, IL). The peptide substrates Ac-DEVD-amino-4-methylcoumarin (Ac-DEVD-amc) and Ac-IETD-amino-4-methylcoumarin (Ac-IETD-amc) were from Bachem (Heidelberg, Germany). The caspase inhibitors zVAD-fmk and DEVD-cmk were from Enzyme Systems Products (Livermore, CA) and Biosyn Diagnostics (Belfast, Northern Ireland), respectively. MitoTracker Red was from Molecular Probes (Leiden, The Netherlands). All salts were from Sigma Chemical (Saint Louis, MO).

Cell culture. Jurkat and A549 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO_2.

Induction of apoptosis and cell viability assay. The adherent A549 cells were detached by incubation in versene for 10 min in 37°C. Harvested cells were centrifuged at 900 rpm for 6 min, washed once with PBS, and resuspended in growth medium without FBS. Cells were plated at 1.8×10^6 cells/ml and treated with MAL or anti-CD95 Abs (CH11) in the absence or presence of inhibitors (20 μM zVAD-fmk or 100 μM DEVD-cmk) for various times at 37°C. After 30 and 90 min of incubation, 5% FBS was added to Jurkat and A549 cells, respectively. The percentage of dead cells was determined using the trypan blue exclusion assay. MAL was purified from human milk as described previously [7] and was dissolved in PBS (10 mg/ml) prior to experiment.

Protease assay. Aliquots containing 0.9×10^6 cells were washed once with ice-cold PBS, resuspended in 25 μl PBS, and then transferred to a 96-well plate. The cells were lysed by transferring the plate to −20°C for at least 30 min. Fifty microliters of freshly prepared substrate buffer (100 mM Hepes, 10% sucrose, 0.1% Chaps, 5 mM DTT, 10^{-6}% NP-40, pH 7.25) containing the substrate Ac-DEVD-amc or Ac-IETD-amc was added per well. The final concentration of Ac-DEVD-amc or Ac-IETD-amc was 33.3 or 13.6 μM, respectively. The enzymatic reactions were carried out at 37°C and the rates of hydrolysis were measured by release of amc from the substrates, using an ELISA reader. Experiments were performed in duplicate and the activity was expressed as change in fluorescence units per minute per 10^6 cells.

Western blotting. Cellular extracts were prepared as follows: cells were pelleted at 1000*g* for 2 min at 4°C and washed in ice-cold PBS. Cell pellets were resuspended in 1× Laemmli's loading buffer and boiled for 4 min. Equal amounts of extracts were subjected to electrophoresis on 7.5 or 12% SDS–polyacrylamide gels. The proteins were then electroblotted to a nitrocellulose membrane, blocked overnight in a high-salt buffer (50 mM Tris–base, 500 mM NaCl) containing 5% dried milk and 1% bovine serum albumin, and probed with the antibodies against p17 (1:5000), PARP (1:2500), lamin B (1:1000), α-fodrin (1:2000), and actin (1:500). Primary antibody binding was detected with a secondary goat anti-mouse (1:10,000) or a goat anti-rabbit (1:10,000) antibody conjugated with horseradish peroxidase and visualized by enhanced chemiluminescence as described in the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Preparation and activation of cytosolic extract. Untreated and MAL-treated cells were washed twice in ice-cold PBS, resuspended at 75×10^6 cells/ml in S-100 buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA), supplemented with protease inhibitors (0.1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, 25 μg/ml calpain I inhibitor, 1 mM DTT), and incubated on ice for 10 min. Cells were centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was subjected to the next centrifugation at 100,000*g* for 1 h and stored at −70°C. Thirty microliters of extract (approx 3 mg protein/ml) was incubated with different concentrations of MAL, or monomeric α-lactalbumin (ALA), in the presence or absence of 1 mM dATP and/or 0.4 μM cytochrome *c* at 30°C for 45 min. The caspase activity was determined by measuring Ac-DEVD-amc cleavage using the protease assay described above. The protein concentrations of the extracts were determined using the BCA protein assay (Pierce). For analysis of cytochrome *c* release, 100 μl of postmitochondrial cytoplasmic extract (10,000*g* supernatant) was mixed with 25 μl 5× Laemmli's loading buffer, boiled for 4 min, and subjected to SDS–PAGE followed by electroblotting. The nitrocellulose membrane was stained with the anti-cytochrome *c* antibody (1:2500).

Isolation of mitochondria. Jurkat cells were pelleted and washed in buffer containing 100 mM sucrose, 1 mM EGTA, and 20 mM Mops. Cell pellet was resuspended in 5% Percoll, 0.01% digitonin, and protease inhibitors and incubated on ice for 10 min, followed by centrifugation at 2500*g* for 5 min. Supernatant was subjected to an additional centrifugation at 10,000*g* for 15 min. Mitochondrial pellet was collected in 300 mM sucrose, 1 mM EGTA, 20 mM Mops, and protease inhibitors and kept at −70°C. Mitochondria (3 mg/ml) were transferred into buffer containing 250 mM sucrose, 10 mM Mops, 5 mM succinate, 3 mM KH_2PO_4, 10 μM EGTA, and 10 mM Tris, pH 7.5, and after incubation at 30°C were centrifuged at 10,000*g* for 15 min. Both pellets and supernatants were subjected to SDS–PAGE and Western blots were analyzed as above.

Colocalization of MAL with mitochondria. A549 cells were incubated with biotin-labeled MAL for 3 h, harvested, and washed with PBS. Cells were fixed in phosphate-buffered paraformaldehyde (4%) for 5 min at room-temperature [10], washed in PBS, and permeabilized with 0.1% Triton X-100. After washing, FITC-conjugated streptavidin (1:100 in 0.1% Triton X-100) was added and the cells were incubated for 30 min at room temperature. After two washes in PBS–Triton X-100

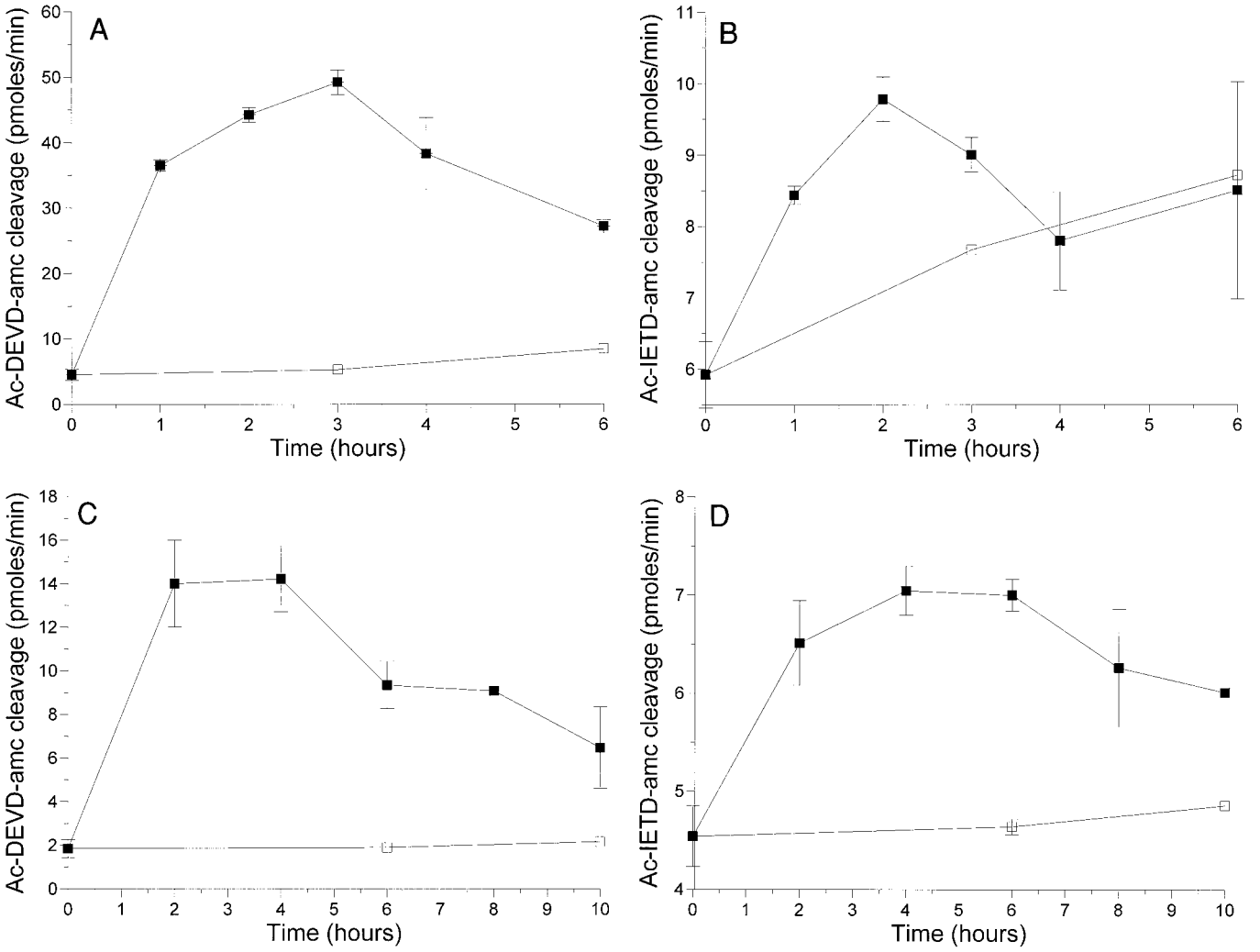

FIG. 1. Activation of caspase-3-like and caspase-6-like enzymes in MAL-treated Jurkat and A549 cells. Untreated (opened symbols) and MAL-treated (closed symbols) Jurkat (A and B) and A549 (C and D) cells were incubated for various times and assayed for the cleavage of the fluorogenic substrates Ac-DEVD-amc (A and C) and Ac-IETD-amc (B and D) as described under Materials and Methods. The data shown are the mean values derived from one of three independent experiments performed in duplicate.

the mitochondria were stained using MitoTracker Red (25 nM in PBS) for 30 min at room temperature. The cells were mounted on a glass slide and analyzed in a Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad Laboratories, Hemel-Hempstead, UK) attached to a Nikon Eclipse 800 upright microscope (Nikon, Japan).

DNA fragmentation. Untreated and treated-with-MAL Jurkat cells were incubated in the presence or absence of zVAD-fmk for 6 h. DNA was isolated from these cells and subjected to agarose gel electrophoresis as described in [7].

RESULTS

Activation of Caspase-3-like and Caspase-6-like Enzymes in MAL-Treated Cells

Caspase activation can be quantified *in vitro* using fluorogenic substrates, which mimic the specific cleavage sites of the different caspases [1]. In this study two cell lines (Jurkat and A549) with different sensitivities to MAL were used. Cells were exposed to MAL at a concentration that gave a 50% reduction in cell viability at 6 h. The concentration of MAL required to reduce the viability of A549 cells by 50% was three times higher than that needed for Jurkat cells. The activation of caspase-3-like and caspase-6-like enzymes was determined by using Ac-DEVD-amc and Ac-IETD-amc substrates, respectively.

The caspase-3-like activity in MAL-treated Jurkat cells was increased approximately 10-fold above the control, and the maximum activity (50 pmol/min/10^6 cells) was found at 3 h (Fig. 1A). MAL-treated A549 cells also showed an increased activity of the caspase-3-like enzymes. This activity was elevated about 7-fold

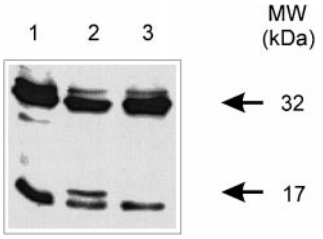

FIG. 2. Processing of caspase-3 during MAL-induced apoptosis in Jurkat cells. Untreated cells (lane 1) or cells treated with MAL (lanes 2 and 3) were also preincubated with 20 μM zVAD-fmk (lane 3). After 4 h incubation, cells were harvested and prepared for Western blot analysis. The membranes were stained with antibodies against the 17-kDa fragment of active caspase-3.

over the control and reached a maximum (14 pmol/min/10^6 cells) after 2 h (Fig. 1C).

It is well known that the 32-kDa procaspase-3 is cleaved upon activation with the formation of 17- and 12-kDa subunits [1]. To further demonstrate the activation of caspase-3 in MAL-treated cells, cell lysates were subjected to Western blot analysis using an antibody against the p17 fragment, which also recognizes the 32-kDa protein. As seen in Fig. 2, treatment of Jurkat cells with MAL resulted in the processing of the 32-kDa procaspase-3 into the 17-kDa subunit of the active protease. Similar results were obtained with A549 cells (data not shown).

MAL treatment stimulated the caspase-6-like proteolytic activity in both Jurkat and A549 cells, but to a much lesser extent compared with the activation of the caspase-3-like enzymes. There was a transient 40% increase of the Ac-IETD-amc cleavage activity after 2 h in Jurkat cells and a more persistent increase of 55% after 4 h of MAL exposure in A549 cells (Figs. 1B and 1D).

Effect of Inhibitors on the Apoptotic Process Induced by MAL

To further confirm the specificity of the intracellular caspase activation and subsequent cleavage of different peptide substrates, cells were pretreated with the broad-range caspase inhibitor zVAD-fmk or the specific inhibitor of caspase-3-like enzymes, DEVD-cmk. As shown in Fig. 3, both agents blocked MAL-induced cleavage of Ac-DEVD-amc substrate in Jurkat and A549 cells. Processing of caspase-3 detected by Western blot technique was also inhibited by zVAD-fmk (Fig. 2). These results demonstrated that caspase-3 was processed and activated in cells exposed to MAL.

It has previously been shown that MAL-induced apoptosis resulted in a massive DNA fragmentation [7]. Incubation of Jurkat cells with zVAD-fmk inhibited the formation of DNA ladder (Fig. 4) and also increased the viability of cells treated with MAL (data not shown), suggesting an important role for caspases in MAL-induced apoptosis.

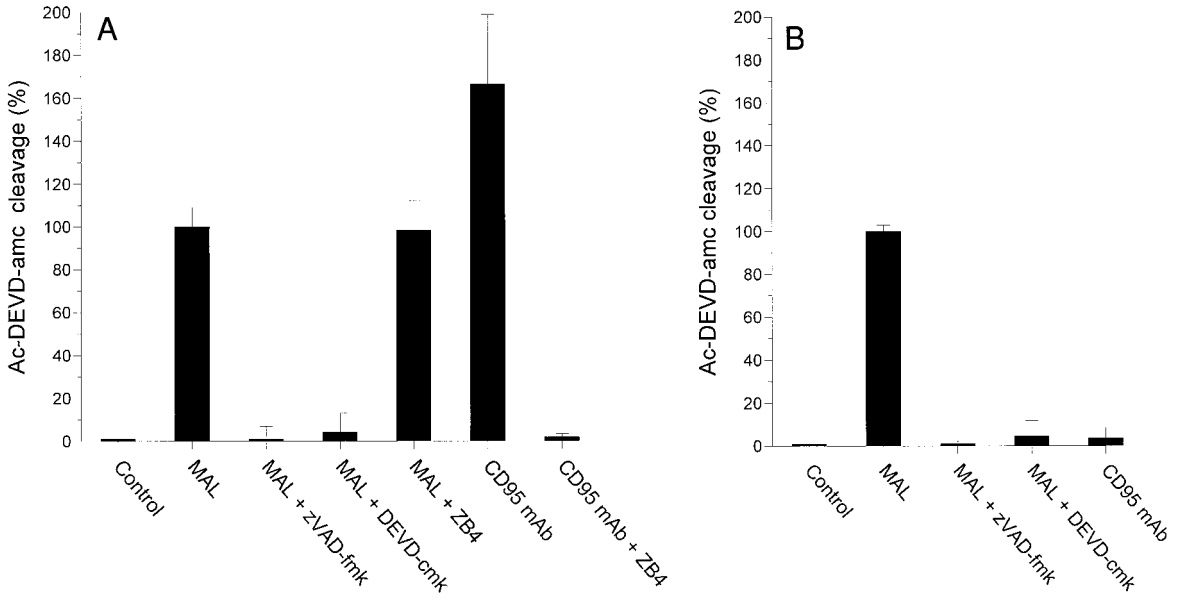

FIG. 3. The effect of inhibitors on the caspase-3-like activity in Jurkat and A549 cells. Jurkat (A) and A549 (B) cells were incubated for 3 h with MAL in the absence (control) or presence of 20 μM zVAD-fmk, 100 μM DEVD-cmk, or 10 μg/ml ZB4. As another control cells were exposed to anti-CD95 mAb (250 ng/ml) alone or in combination with 10 μg/ml ZB4 for 2 h. The cleavage of Ac-DEVD-amc was measured as described under Materials and Methods and expressed as the percentage of the amc released in MAL-treated cells. The values are means $\pm$ standard deviations of three experiments performed in triplicate.

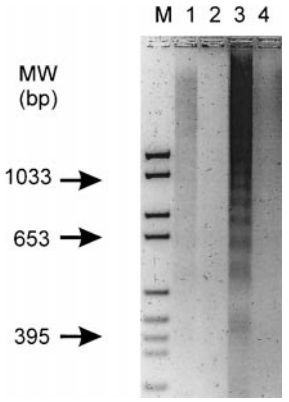

FIG. 4. Inhibitory effect of zVAD-fmk on MAL-induced DNA fragmentation. Untreated (lanes 1 and 2) or MAL-treated (lanes 3 and 4) Jurkat cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of zVAD-fmk for 6 h and extracted DNA was subjected to electrophoresis on agarose gel as described under Materials and Methods.

Cleavage of Intracellular Target Proteins in MAL-Treated Cells

Cleavage of the target proteins PARP, lamin B, and α-fodrin is a consequence of caspase-3 and caspase-6 activation. Jurkat and A549 cells were treated with MAL for longer periods of time (6 and 11 h, respectively), and the cleavage of the above-mentioned proteins was examined by Western blot analysis. As shown in Fig. 5, the activation of caspases in Jurkat cells by MAL treatment resulted in cleavage of PARP, lamin B, and α-fodrin with formation of 85-, 46-, and 120-kDa fragments, respectively. In A549 cells both PARP and α-fodrin were efficiently proteolyzed, but only a small fraction of lamin B was cleaved at the investigated time point.

The broad-range caspase inhibitor zVAD-fmk prevented MAL-induced proteolysis of PARP and lamin B in Jurkat cells (Fig. 5). It is known that native α-fodrin (240 kDa) is cleaved during apoptosis with the formation of two fragments of 150 and 120 kDa [11]. The smaller, 120-kDa fragment is produced by active caspase-3. Indeed, zVAD-fmk completely inhibited the formation of the 120-kDa breakdown product of α-fodrin (Fig. 5). Pretreatment of A549 cells with zVAD-fmk also had an inhibitory effect on the degradation of PARP, lamin B, and α-fodrin (Fig. 5).

Lack of Involvement of CD95-Mediated Pathway in MAL-Induced Apoptosis

To investigate the possible involvement of a CD95-mediated pathway in the activation of caspases by MAL treatment, Jurkat cells were pretreated with an antagonistic anti-CD95 receptor antibody, ZB4. This pretreatment did not have any inhibitory effect on the caspase-3-like activity induced by MAL (Fig. 3A). ZB4 did not have any significant effect on the MAL-induced proteolysis of procaspase-3, PARP, lamin B, or α-fodrin in Jurkat cells (data not shown). A549 cells were insensitive to treatment with agonistic CD95 mAbs (Fig. 3B) due to a lack of translocation of the CD95 receptor to the cell surface [12]. In concordance, there was no effect of ZB4 on apoptosis induced by MAL in these cells (data not shown).

Lack of Direct Activation of Caspase-3-like Enzymes in Cytosolic Extracts by MAL

Soon after administration to intact cells, MAL appears in the cytoplasm [9] where the apoptotic proteolytic machinery is constitutively present and is under tight control of inhibitory factors (for review see [13]). In cytosol, MAL may directly influence caspase activation or interact with inhibitory proteins and thereby indirectly activate caspases in an apoptosome complex.

Cytosolic extract from untreated cells is a well-known *in vitro* model for the activation of caspases [3–6]. In our study, the activation of caspase-3-like enzymes was monitored in extracts from Jurkat and A549 cells after incubation with different compounds (Table 1). Neither MAL, in any concentration, nor monomeric, native α-lactalbumin was able to induce caspase-3-like activity in cytosolic extracts in the pres-

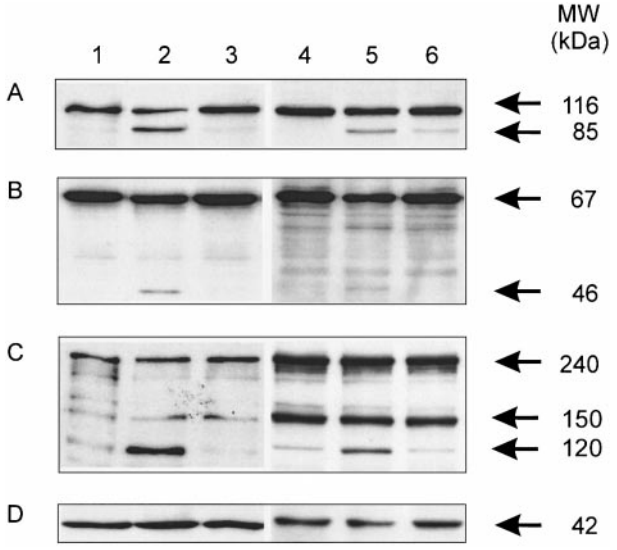

FIG. 5. Cleavage of death substrates in Jurkat and A549 cells undergoing MAL-induced apoptosis. Jurkat (lanes 1–3) and A549 (lanes 4–6) cells were either untreated (lanes 1 and 4) or treated with MAL (lanes 2, 3, 5, and 6) in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 20 μM zVAD-fmk for 6 h (Jurkat cells) or 11 h (A549 cells). Protein extracts were prepared from these cells and were fractionated by SDS–PAGE and transferred to nitrocellulose membranes as described under Materials and Methods. The membranes were stained with antibodies against PARP (A), lamin B (B), α-fodrin (C), and actin (D).

TABLE 1

In Vitro Activation of Caspase-3-Like Enzymes in Cytosolic Extracts

Sample	DEVD-amc cleavage (pmol/min) Jurkat	A549
1. Control	0.98	1.38
2. Cytochrome *c* + dATP	12.3	2.27
3. dATP	0.41	1.42
4. ALA 0.3 mg/ml + dATP	0.95	1.28
5. MAL 0.3 mg/ml	0.98	1.12
6. MAL 0.01 mg/ml + dATP	0.91	1.25
7. MAL 0.03 mg/ml + dATP	1.00	1.18
8. MAL 0.1 mg/ml + dATP	0.81	1.33
9. MAL 0.3 mg/ml + dATP	0.83	1.24
10. MAL 0.1 mg/ml + cytochrome *c* + dATP	4.59	2.02

Note. Cytoplasmic extracts were prepared from Jurkat and A549 cells as described under Materials and Methods. The extracts were incubated with various concentrations of MAL or monomeric α-lactalbumin (ALA) in the absence or presence of 1 mM dATP and/or 0.4 μM cytochrome *c* at 30°C for 45 min. The samples were assayed for Ac-DEVD-amc cleavage activity. These results are representative of two independent experiments.

ence or absence of dATP. This suggests that MAL cannot directly activate the caspase cascade in the cytosol. The addition of dATP and cytochrome *c* to extracts from the tested cell lines resulted in caspase-3-like enzyme activation. When MAL was added to cytosolic extracts treated with dATP and cytochrome *c*, it lowered the activation of caspase-3-like enzymes in Jurkat cells (Table 1).

MAL-Induced Cytochrome c Release

To determine if MAL causes cytochrome *c* release from the mitochondria, postmitochondrial extracts from control and MAL-treated Jurkat cells were prepared (see Materials and Methods) and analyzed for the presence of cytochrome *c* by Western blotting. As shown in Fig. 6, cytochrome *c* appeared in the cytosol from Jurkat cells 1 h after treatment with MAL and the level of cytochrome *c* increased with time of incubation. After being stripped, the membranes were reprobed with antibodies against p17 (subunit of active caspase-3) and actin (to control the amount of protein loaded in each lane). The 17-kDa fragment of active caspase-3 appeared concomitantly with the release of cytochrome *c* (Fig. 6).

In order to investigate the mechanism of MAL-induced cytochrome *c* release two sets of experiments were undertaken. First, cells were incubated with biotin-labeled MAL and confocal microscopy study revealed the punctate localization of MAL in the cytoplasm and nucleus (Fig. 7A). The double staining with MitoTracker Red, a specific mitochondrial probe, showed that MAL in cytoplasm colocalized with mitochondria (Fig. 7C). Second, to confirm a direct effect of MAL on mitochondria, isolated mitochondria were incubated with MAL and the release of cytochrome *c* was investigated. Incubation of mitochondria, without additions, was not associated with any leakage of cytochrome *c* (Fig. 7D, lane 1). In the presence of 0.1% Triton X-100 a significant part of cytochrome *c* appeared in the medium (Fig. 7D, lane 2). Similarly, the incubation of mitochondria with MAL resulted in the concentration-dependent release of cytochrome *c* (Fig. 7D, lanes 3 and 4). Since the native monomeric ALA does not induce apoptosis in any tested cells, the isolated mitochondria were also incubated with ALA, as a negative control. In fact, ALA even at high concentration did not induce cytochrome *c* release to an extent similar to that of MAL (Fig. 7D, lane 5).

DISCUSSION

Diverse physiological and pathological stimuli can induce apoptosis by distinct signaling pathways that converge in a common program of cell suicide. The activation of the caspase family of proteases has been detected in numerous cell systems and appears to function as a pathway through which apoptotic mechanisms operate. Upon apoptotic triggering, a hierarchy of caspases is believed to become activated in a process in which more proximal caspases cleave and activate downstream caspases thereby giving rise to a proteo-

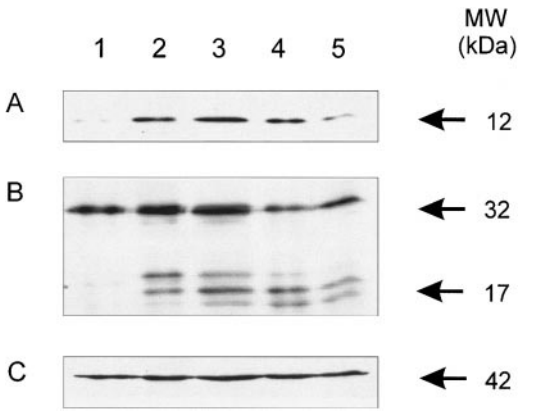

FIG. 6. Release of cytochrome *c* in MAL-treated Jurkat cells. Untreated cells (lane 1) or cells treated with MAL were incubated for 1 (lane 2), 2 (lane 3), 4 (lane 4), or 6 h (lane 5). Protein extracts were prepared from postmitochondrial supernatants isolated from these cells, and after Western blotting nitrocellulose membranes were analyzed for the presence of cytochrome *c* (A) and the activation (cleavage) of the native 32-kDa protein of procaspase-3 (B) as described under Materials and Methods. Membranes were subsequently reprobed with antibodies against actin to control for equal loading (C). The blots are representative of three independent experiments.

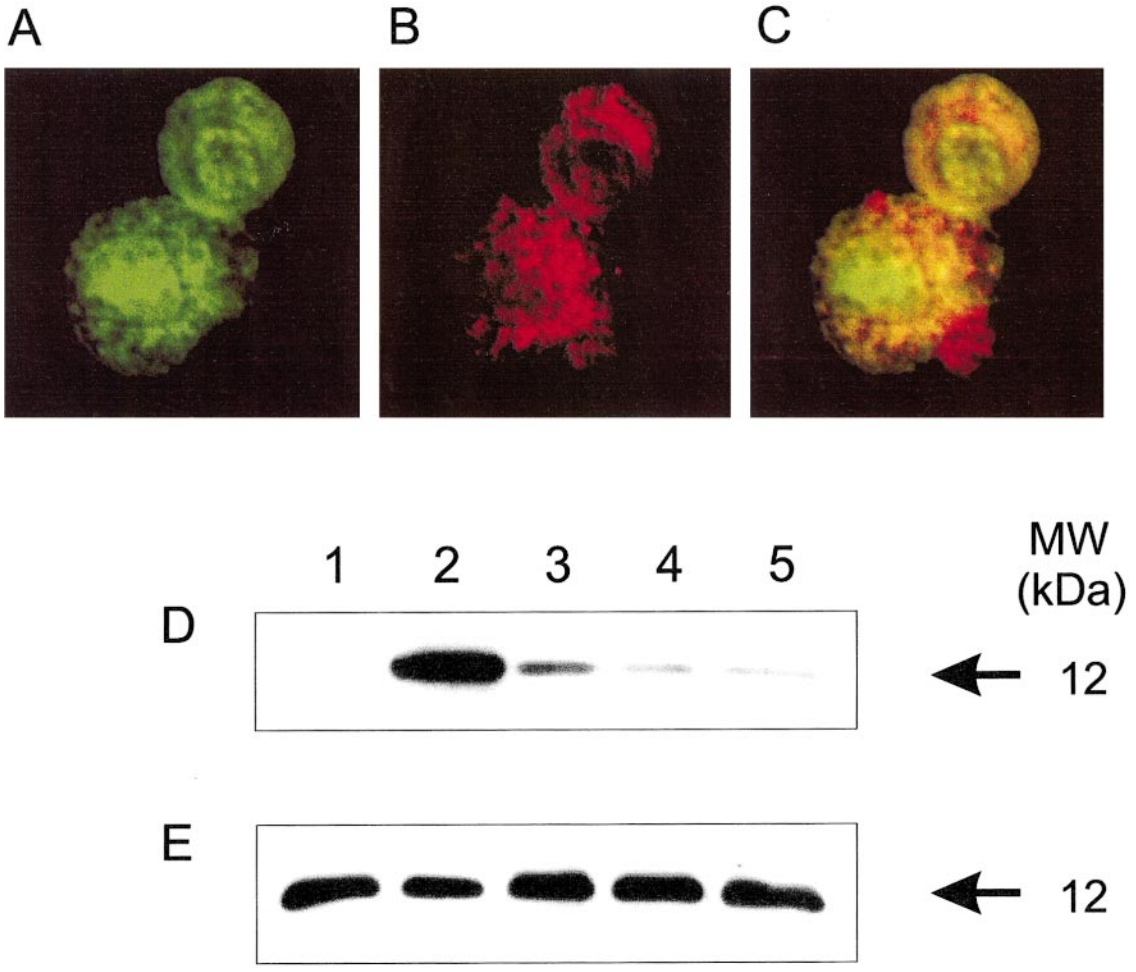

FIG. 7. Interaction of MAL with mitochondria. Cells were incubated with biotin-labeled MAL, fixed, permeabilized, and incubated with FITC-streptavidin. Mitochondria were stained with MitoTracker Red. The pictures show staining of MAL (green) (A) and staining of mitochondria (red) (B), and a merged image shows the colocalization of MAL and mitochondria (yellow) (C). Isolated mitochondria were incubated alone (lane1) or in presence of Triton X-100 (lane 2), 10 and 5 μg/ml MAL (lanes 3 and 4, respectively), or 10 μg/ml ALA (lane 5), and after centrifugation both supernatant (D) and pellet (E) were subjected to SDS–PAGE. Membranes were stained with anti-cytochrome *c* mAbs as described under Materials and Methods.

lytic cascade that serves to amplify the death signal. Here, we investigated the involvement of the cellular proteolytic machinery in apoptosis induced by MAL and compared the profile of caspase activation in two cell lines with different sensitivities to MAL.

Caspase-3 and caspase-6 are the most commonly activated caspases during the execution phase of apoptosis [14]. In this study MAL treatment of Jurkat and A549 cells caused a modest increase of the caspase-6-like enzymatic activity and a more pronounced induction of the caspase-3-like activity. There are several possible explanations for these results. One is that caspase-6-like proteases may act upstream of the caspase-3-like enzymes in the proteolytic cascade induced by MAL and that a low activity of this enzyme may be sufficient to cleave and activate procaspase-3. This has been shown to occur in intestinal epithelial cells that are shed after reaching the lumen. In these cells the activation of procaspase-6 preceded the activation of procaspase-3 [15]. It has also been demonstrated that caspase-3-like proteases can be cleaved by caspase-6-like proteases (to which caspase-9 also belongs) [5, 16, 17]. Thus, it is likely that the same event takes place *in vivo.* Furthermore, it is well recognized that several proteins localized in cytoplasm, membranes, and nuclei of apoptotic cells are targets for caspase-3 and that the only known target for caspase-6 is lamin [18]. The relatively low caspase-6-like activity may be sufficient to achieve the degradation of protein substrates.

In addition, the caspase-3-like activity in Jurkat cells was about three times higher than in the A549 cells upon MAL treatment. Since the Jurkat cells were more sensitive to killing by MAL than the A549 cells,

this may suggest that the sensitivity of cells to undergo apoptosis is related to the intrinsic level of activation of caspases. This difference in activation of caspase-3-like activity could also be due to differences in the regulation of the apoptotic pathway with lower threshold levels in cells that more readily undergo apoptosis.

To participate in the apoptotic process, the activated caspases should cleave specific target proteins. Indeed, two substrates for caspase-3, PARP and α-fodrin, were cleaved and their cleavage as well as the activity of caspase-3 was inhibited by the pancaspase inhibitor, zVAD-fmk. Lamin is reported to be cleaved by caspase-6 [18]. Our data show that lamin B was degraded in MAL-treated cells, which further implies the involvement of caspase-6-like enzymatic activity in apoptosis induced by MAL.

There are three possible pathways by which MAL may activate the caspase cascade. First, MAL binds to the plasma membrane and may induce caspase activation via a receptor-mediated sequence of events. One of the best characterized receptor-mediated systems involved in apoptosis is CD95 (Fas/APO-1). However, pretreatment of Jurkat cells with an antagonistic anti-CD95 antibody, ZB4, did not have any significant effect on the MAL-induced DEVD-cleavage activity or degradation of target proteins. This suggests that a CD95 receptor-mediated pathway is not involved in MAL-mediated killing. However, these data do not exclude the possibility that any other receptor-mediated pathway might be involved in the apoptosis induced by MAL.

Second, MAL has been shown to accumulate in the nuclei of sensitive cells [9]. There is a possibility that caspase activation in MAL-treated cells requires signals produced by interactions at the nuclear level. This approach is used by glucocorticoid hormones that upon binding of their cytosolic receptors are translocated to the nucleus, where the signal for apoptosis induction is produced [19].

Third, as MAL is internalized, it may have the potential to directly influence caspase activation in the cytoplasm or interact with inhibitory proteins and thereby indirectly activate caspases by the engagement of the apoptosome pathway. The latter involves mitochondrial events that result in the release of cytochrome *c*, which, as part of the apoptosome complex, participates in the caspase activation. However, experiments with S-100 supernatant isolated from different cell lines showed that MAL was not able to activate caspase-3-like enzymes directly in cytosol. Moreover, the presence of MAL in the incubation mixture in fact lowered caspase activation in S-100 supernatants induced with cytochrome *c* and dATP. These data are in agreement with observations that the activation of caspases requires interaction of very specific molecules and that the presence of "unusual proteins" as well as high salt concentrations do suppress the enzyme activation [20, 21]. However, it is conceivable that MAL via interaction with any cytoplasmic compartment, such as ER or mitochondria, can induce the activation of the proteolytic cascade.

In different experimental models, other than receptor-mediated killing, a lack of cytochrome *c* in the cytosol is the primary limiting factor for caspase activation [22, 23]. In our experiments, cytochrome *c* was indeed released from the mitochondria into the cytosol of MAL-treated cells. Incubation of cells with biotin-labeled MAL showed colocalization of MAL and mitochondria. Moreover, in the presence of MAL cytochrome *c* was released from isolated mitochondria. Thus, it is likely that the cytochrome *c* release in this experimental model is mediated by the direct interaction of MAL with mitochondria.

In conclusion, we have shown that MAL-induced apoptosis involves activation of the proteolytic cascade of caspases and that the release of cytochrome *c* is an important step in the initiation and/or amplification of the caspase cascade.

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REFERENCES

1. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. *J. Biol. Chem.* **272,** 17907–17911.
2. Salvesen, G. S., and Dixit, V. M. (1997). Caspases: Intracellular signaling by proteolysis. *Cell* **91,** 443–446.
3. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. *Cell* **86,** 147–157.
4. Yang, J., Liu, X., Bhalla, K., Naekyung, K., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked. *Science* **275,** 1129–1132.
5. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91,** 479–489.
6. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* **90,** 405–413.
7. Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. (1995). Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA* **92,** 8064–8068.

8. Svensson, M., Sabharwal, H., Håkansson, A., Mossberg, A-K., Lipniunas, P., Leffler, H., Svanborg, C., and Linse, S. (1999). Molecular characterization of α-lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* **274,** 6388–6396.
9. Håkansson, A., Andreasson, J., Zhivotovsky, B., Karpman, D., Orrenius, S., and Svanborg, C. (1999). Multimeric α-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp. Cell Res.* **246,** 451–460.
10. Sander, B., Andersson, J., and Andersson, U. (1991). Assessment of cytokines by immunofluorescence and the paraformaldehyde–saponin procedure. *Immunol. Rev.* **119,** 65–92.
11. Vanags, D. M., Pörn-Ares, M. I., Coppola, S., Burgess, D. H., and Orrenius, S. (1996). Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem.* **271,** 31075–31085.
12. Nambu, Y., Hughes, S. J., Rehemtulla, A., Hamstra, D., Orringer, M. B., and Beer, D. G. (1998). Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinomas. *J. Clin. Invest.,* **101,** 1102–1110.
13. Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: Enemies within. *Science* **281,** 1312–1316.
14. Faleiro, L., Kobayashi, R., Fearnhead, H. R., and Lazebnik, Y. (1997). Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16,** 2271–2281.
15. Grossmann, J., Mohr, S., Lapetina, E. G., Fiocchi, C., and Levine, A.,D. (1998). Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. *Am. J. Gastrointest. Liver Physiol.* **37,** G1117–G1124.
16. Orth, K., O'Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996). Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. *J. Biol. Chem.* **271,** 20977–20980.
17. Liu, X., Kim, C. N., Pohl, J., and Wang, X. (1996). Purification and characterization of an interleukin-1β-converting enzyme family protease that activates cysteine protease P32 (CPP32). *J. Biol. Chem.* **271,** 13371–13376.
18. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996). The CED-3/ICE-like Mch-2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* **271,** 16443–16446.
19. Harris, A. C., Tomkins, G. M., and Cohn, M. (1971).Glucocorticoid receptors in lymphoma cells in culture. Relationship to glucocorticoid killing activity. *Science* **171,** 189–191.
20. Chou, J. J., Matsuo, H., Duan, H., and Wagner, G. (1998). Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. *Cell* **94,** 171–180.
21. Hampton, M. B., Zhivotovsky, B., Slater, A. F. G., Burgess, D. H., and Orrenius, S. (1998). Importance of the redox state of cytochrome *c* during caspase activation in cytosolic extracts. *Biochem. J.* **329,** 95–99.
22. Reed, J. C. (1997). Cytochrome *c:* Can't live with it—Can't live without it. *Cell* **91,** 559–652.
23. Han, Z., Li, G., Bremner, T. A., Lange, T. S., Zhang, G., Jemmerson, R., Wyche, J. H., and Hendrickson, E. A. (1998). A cytosolic factor is required for mitochondrial cytochrome *c* efflux during apoptosis. *Cell Death Differ.* **5,** 469–479.

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