Heavy Membrane-Associated Caspase 3: Identification, Isolation, and Characterization

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Received May 2, 2000; Revised Manuscript Received September 26, 2000

ABSTRACT: Heavy membrane preparations from 697 lymphoblastoid cells contain a tightly bound caspase zymogen. This heavy membrane-bound procaspase can be efficiently liberated from membrane preparations using detergents. Alternatively, the procaspase can be rapidly processed and activated from membrane preparations by caspase-1 without detergents. The activated caspase-3 was purified using affinity chromatography and characterized by amino acid sequencing and inhibitor specificity analysis. The sequence indicates that this heavy membrane bound caspase is caspase-3. The kinetic properties and inhibitor binding specificity also show that this purified caspase is enzymologically indistinguishable from cytoplasmic or recombinant caspase-3. However, the N-termini of activated heavy membrane-bound and cytoplasmic caspase-3 are slightly different; peptide sequencing data indicate that the heavy membrane caspase-3 begins at Lys 14, whereas the cytoplasmic enzyme begins at Ser 10. Implications of this structural difference are discussed.

Caspases are a family of intracellular cysteine proteases that are essential effectors of apoptosis (*1*−*4*). Active caspases are tetrameric enzymes composed of two large (18 kDa) and two small (12 kDa) subunits. The active site of caspase appears to locate between the large and the small subunits (*5*−*7*). Caspases cleave proteins immediately after aspartic acid (P1) residues; however, the three residues prior to the aspartic acid residue (P2−P4) are also critical in determining the substrate specificity (*8*). Not surprisingly, caspases can efficiently and specifically cleave certain synthetic tetrapeptide substrates, such as Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC)[1] (*9*). In a similar vein, caspases are potently inhibited by tetrapeptide aldehydes such as Asp-Glu-Val-Asp-aldehyde (DEVD-aldehyde) (*9*, *10*).

In cells, caspases are synthesized as inactive zymogens (procaspases). Procaspases are converted to active caspases when they are cleaved at specific aspartic acid residues (*3*). Cleavage at these sites causes the liberation of the mature large and small subunits from the precursor. It has been postulated that during apoptosis, upstream apical procaspases are activated by a cofactor-facilitated autoprocessing mechanism (*11*). In this scheme, activation of the apoptotic pathway causes a recruitment of the apical procaspases to complexes such as the death induced signaling complex (DISC) at the plasma membrane (*12*, *13*) or the Apaf-1/cytochrome apoptosome complex in the cytoplasm (*14*). Once bound to the complex, the upstream procaspases autoprocess to become active enzyme. The activated caspases in turn proteolytically activate downstream procaspases, such as procaspase-3. Once activated, downstream caspases execute cells by cleaving specific molecular targets that are essential for cell viability or by activating proapoptotic factors (*3*, *15*, *1*6).

Caspase-3 is a downstream "executioner" caspase thought to cleave a number of important cellular proteins involved in DNA replication, DNA repair, RNA splicing, protein phosphorylation, and chromosomal fragmentation during apoptosis (*1*, *15*, *16*). This enzyme is synthesized as a 32 kDa zymogen that is processed to mature 20/17 kDa and 12 kDa subunits by cleavage at Asp 9, Asp 28, and Asp 175 (*9*, *17*, *18*). Procaspase-3 can be activated by a number of proteases involved in apoptosis including caspases-1, -8, -9, and -10, as well as the serine protease granzyme B (*18*−*21*). Activated caspase-3 has been isolated from the cytoplasm of THP-1 (*9*), Jurkat (*22*), HeLa, and liver cells (*23*). Immunocytochemical experiments also indicate that procaspase-3 is primarily a cytoplasmic protein (*24*−*27*), while activated caspase-3-like enzyme is found in the cytoplasm and the nucleus (*28*). However, it has been reported that procaspase-3 is also localized in the mitochondrial intermembrane space (*29*).

We have also recently described the identification of a heavy membrane-associated caspase-3-like activity from 697 lymphoblastoid cells whose activation is regulated by the antiapoptotic gene Bcl-2 (*21*). In this paper, we describe the isolation and biochemical characterization of that activity.

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[1] Abbreviations: DEVD-AMC, acetyl-Asp−Glu−Val−Asp-aminomethylcoumarin; DEVD-aldehyde, acetyl-Asp−Val−Val−Asp-aldehyde; YVAD-aldehyde, acetyl-Tyr−Val−Ala−Asp-aldehyde; DTT, dithiothreitol; 697-neo cells, 697 cells stably infected with a retrovirus expressing the neomycin resistance gene; 697-Bcl-2 cells, 697 cells stably infected with a retrovirus expressing human bcl-2 cDNA; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; NBT, nitro blue tetrazolium; TBS, Tris-buffered saline; CMC, critical micelle concentration; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MES, 2-[*N*-morpholino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-diaminetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; PVDF, poly(vinylidene difluoride).

10.1021/bi001007w CCC: $19.00
Published on Web 11/30/2000

MATERIALS AND METHODS

Cell Lines and Cell Production. 697 human lymphoblastoid cells stably infected with a retroviral expression construct containing bcl-2 cDNA (697-Bcl-2 cells) or a control neomycin resistance gene (697-neo cells) (*40*), obtained from Dr. John Reed, Burnham Institute, were used in these studies. The cells were maintained in mid-log phase growth in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 0.2 mg/mL G-418 (Gibco, Gaithersburg, MD), and 0.1 mg/mL penicillin/streptomycin (Irvine Scientific).

Subcellular Fractionation. 697 cells were harvested from 2-L cultures (≈ 2 billion cells total) by centrifugation and washed twice in cold phosphate buffered saline. The washed cells were resuspended in cold hypotonic buffer (10 mM Na-HEPES, 5 mM $MgCl_2$, 42 mM KCl, pH 7.4) supplemented with 2 mM DTT (Sigma, St. Louis, MO) and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 5 μg/mL aprotinin, 0.1 mM EDTA, and 0.1 mM EGTA, all purchased from Sigma, St. Louis, MO) to a density of $\approx 3 \times 10^8$ cells/mL. The samples were incubated on ice for 30 min at which time the cells were lysed using 30−40 strokes with a Dounce homogenizer. The sample was centrifuged twice for 10 min at 500*g*, 4 °C, to separate the nuclei. The nuclear pellet was washed twice in the same buffer supplemented with 1.6 M sucrose. The 500*g* supernatant was then centrifuged at 14000*g* for 30 min at 4 °C to pellet the heavy membranes. The heavy membranes were washed 3 times with 1.5 mL cold hypotonic buffer containing protease inhibitors and DTT. The washed membranes were resuspended in hypotonic buffer so that the total protein concentration was approximately 2 mg/mL. This yielded the heavy membrane fraction. The sample was either flash frozen or used immediately for enzymatic measurements without freezing. The 14000*g* supernatant was centrifuged again at 100000*g* for 30 min at 4 °C, yielding supernatant (cytoplasmic fraction). Protein concentrations were measured using Protein Assay Kit II from BioRad with bovine serum albumin as the calibration standard.

Enzyme Activity and Inhibition Studies. Caspase activity was measured by mixing 50 μL of an enzyme-containing fraction and 200 μL of 25 μM Asp−Glu−Val−Asp-aminomethylcoumarin (DEVD-AMC) substrate in ICE buffer (20 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT, pH 7.5) in duplicate Cytoplate wells. AMC product formation was monitored by the increase in fluorescence (ex = 360 nm, em = 460 nm) over 1 h at 30 °C using the CytoFluor 4000 plate reader (Perseptive Biosystems, Framingham, MA). For kinetic studies, the rate of product formation was measured throughout the substrate range 1−200 μM. K_M values were calculated from these measurements using the equation:

$$\Delta FL/\Delta t = (\Delta FL/\Delta t)_{MAX}/(1 + K_M/[S])$$

where $\Delta FL/\Delta t$ is the observed initial rate of fluorescence change at substrate concentration [S] and $(\Delta FL/\Delta t)_{MAX}$ is the initial rate of fluorescence change at saturating substrate concentrations. For inhibition studies, 50 μL of enzyme was pretreated with 150 μL of inhibitor for 30 min at room temperature prior to the addition of 50 μL of 100 μM substrate solution. Inhibitor IC_{50} values were determined using the equation:

$$\Delta FL/\Delta t = (\Delta FL/\Delta t)_o/(1 + [I]/IC_{50})$$

where $\Delta FL/\Delta t$ is the observed initial rate of fluorescence change at inhibitor concentration [I], and $(\Delta FL/\Delta t)_o$ is the initial rate of fluorescence change for the uninhibited enzyme.

Caspase Activation. Heavy membrane samples were diluted to 1 mg/mL in hypotonic buffer containing 5 mM DTT. Caspase activation was induced by adding either 60−600 ng/mL (final concentration) recombinant murine caspase-1 (in bacterial lysate) or buffer and incubating the samples for 60 min at 30 °C. After the activation period, the heavy membrane pellet was separated from the supernatant by centrifugation for 10 min at 14000*g* at 4 °C. In all experiments, the observed DEVD-AMC cleaving activities in the supernatants were corrected for the activity of the exogenous caspase-1, which was negligible as compared to the total activity. In some experiments, caspase-1 activity was rapidly quenched at specific time points by the addition of one-tenth volume of 3 μM YVAD-aldehyde (Bachem Bioscience, King of Prussia, PA) prior to centrifugation. Detergent and buffer effects were analyzed by incubating the membranes on ice with one-tenth volume of 0−10% detergent in hypotonic buffer. After 30 min, the membranes were collected by centrifugation (10 min at 14000*g*) and resuspended in an identical volume of hypotonic buffer prior to activation with caspase-1.

Production of Recombinant Caspase-3 and p35 Proteins. BL21 (DE3) cells harboring a plasmid containing the cloned human caspase-3 cDNA (*17*) (provided by Dr. E. Alnemri, Thomas Jefferson University) was ligated into the *Bam*HI/*Xho*I sites of pET21b (Novagen, Madison, WI) and were grown in 1-L induction medium (20 g/L tryptone, 10 g/L yeast extract, 6 g/L NaCl, 3 g/L Na_2HPO_4, 1 g/L KH_2PO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4) containing 0.1 mg/mL ampicillin at 37 °C. When the culture density reached $A_{600} = 1$, IPTG (Sigma) was added to a concentration of 1 mM and the culture was incubated at 25 °C for 3 h. The cells were harvested by centrifugation at 2000*g* for 15 min at 4 °C. The cells were lysed using one freeze−thaw cycle in 100 mL of binding buffer (20 mM Tris-Cl, 500 mM NaCl, 5 mM imidazole, and 0.1% Triton X-100) with 0.1 mg/mL lysozyme (InovaTech, Abbottsford, BC, Canada). Cell debris was removed from the sample by centrifugation at 20000*g*, for 30 min at 4 °C. The lysed cells were treated just prior to centrifugation with $MgCl_2$ and DNase I to reduce viscosity. The supernatant was filtered through a 0.45 μm syringe filter and loaded onto a 1 mL Ni^{2+} charged HiTrap Chelation column (Amersham-Pharmacia, Uppsala, Sweden) at a 1 mL/min flow rate. The column was washed at 1 mL/min with 10 mL of binding buffer followed by 10 mL of binding buffer containing 60 mM imidazole (Sigma, St. Louis, MO). The caspase-3 protein was eluted from the column using a 30 mL linear gradient of imidazole (60−500 mM). P35 protein [early p35 protein from *autographa californica* nuclear polyhedrosis virus (*30*)] was produced and purified using an identical procedure.

Production of Recombinant Caspase-1. Recombinant murine caspase-1 was expressed using BL21 (DE3) pLys S cells

harboring pET3ap30mICEFLAG plasmid (a generous gift of Drs. H. R. Horvitz and Ding Xue, MIT) which contains the p30 caspase-1 cDNA inserted into the *Nde*I/*Bam*HI sites of the pET3a expression vector (Novagen). A 3-L culture was grown at 37 °C in LB medium containing 0.1 mg/mL ampicillin and 0.025 mg/mL chloramphenicol. When the culture reached a density of $A_{600} = 1.0$, IPTG was added to 1 mM and the culture was shaken at 25 °C for 3 h. The cells were collected by centrifugation at 2000*g* for 15 min at 4 °C and resuspended in 100 mL of cold buffer containing 25 mM TrisCl, pH 8.0, 25 mM KCl, 0.1% Triton X-100, and 0.1 mg/mL lysozyme. The cells were lysed using one freeze/thaw cycle and the lysate was clarified by treating the sample with 0.02 mg/mL DNase I and 0.5 mM $MgCl_2$ (Sigma) for 60 min and then centrifuging at 20000*g* for 30 min at 4 °C to remove cell debris. The p30 caspase-1 gene was also inserted into the *Nde*I/*Xho*I sites of pET21b plasmid and the enzyme was produced and purified using the procedure utilized for the p35 and caspase-3 proteins.

Affinity Purification of Membrane Caspase. Heavy membranes were prepared from frozen 697 cell pellets containing ≈100 billion cells total as described above. The heavy membranes were resuspended in hypotonic buffer (to a density of $\approx 7 \times 10^8$ cells/mL) and activated with recombinant caspase-1 for 90 min at 30 °C. Supernatant from activated heavy membranes was treated with 300 nM YVAD-aldehyde for 15 min such that caspase-1 activity is completely inhibited. The resultant sample was filtered through a 0.45 μm acrodisc syringe filter and subsequently loaded at 1 mL/min onto a 0.8-mL streptavidin agarose (Sigma, St. Louis, MO) column charged with biotinyl-DEVD-aldehyde affinity reagent (Peptides International, Louisville, KY). The column was washed three times with 12 mL of hypotonic buffer supplemented with 0.15 M NaCl to remove nonspecifically bound material. After the resin was washed with 3 mL of hypotonic buffer, the resin was resuspended with 0.45 mL of additional hypotonic buffer and split into two portions. Protein was eluted from the larger portion (0.65 mL) under denaturing conditions by treating the resin with 0.6 mL of 0.4% SDS in 20 mM MES buffer, pH 5.5, for 15 min at 65 °C. After elution, the sample was lyophilized prior to SDS−PAGE analysis. The smaller portion (0.15 mL resin) was treated with 2 mL of 100 mM hydroxylamine, 20 mM oxidized glutathione, pH 7.5, for 8 h to elute the caspase under nondenaturing conditions (*8*). After elution, the hydroxylamine/glutathione was removed by passing the sample through a PD10 desalting column (Amersham-Pharmacia) equilibrated with ICE buffer + 50 mM NaCl. The caspase was then rapidly reactivated by dilution in buffer containing 30 mM DTT.

Immunoblot and N-Terminal Sequence Analysis. Caspase samples were reduced/denatured with β-mercaptoethanol/SDS buffer, heated to 100 °C for 5 min, and briefly microfuged to remove any insoluble material. Samples were electrophoresed using 15% SDS−PAGE Ready Gels (Bio-Rad, Hercules, CA) and then transferred to PVDF membrane (BioRad). Membranes were blocked in TBS/0.05% Tween 20 (TBST) + 3% BSA (Sigma, St. Louis, MO). Blots were incubated in 1 μg/mL polyclonal anticaspase-3 antibody (*31*) diluted in TBST/3% BSA for 1 h. Following two washes in TBST, blots were incubated for 1 h in 1:15000 dilutions of alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Tropix) in TBST/3% BSA. Blots were then washed twice with TBST and twice in alkaline phosphatase buffer, pH 9.5. The immunoreactive bands were visualized using the colorimetric BCIP/NBT alkaline phosphate substrate (Amersham-Pharmacia).

For N-terminal sequencing analysis, the caspase bands were transferred to PVDF, visualized using Coomassie R-250 stain, dried, and then excised. The PVDF bound band was then analyzed by N-terminal microsequence analysis by Dr. Andy Brauer, Ariad Pharmaceuticals.

RESULTS

Proteolytic Activation of Heavy Membrane Procaspase from 697 Cells using Caspase-1. Heavy membrane fractions from 697 lymphoblastoid cells contain a caspase-3-like proenzyme (*21*). This proenzyme slowly activates at room temperature. Alternatively, the membrane procaspase can be rapidly activated by recombinant caspase-1 (*21*). To characterize the activation and biochemical properties of the membrane caspase, we prepared extensively washed heavy membranes from 697 cells. Heavy membranes fractions are highly enriched in mitochondria and devoid of cytoplasmic or nuclear material (*21*). The isolated membranes contain very little caspase activity (Figure 1, panel A). Treatment with recombinant caspase-1 causes a rapid, robust activation of the membrane caspase, as indicated by the emergence of DEVD-AMC hydrolysis activity (Figure 1, panel A). The DEVD-AMC hydrolysis activity does not emanate from caspase-1, since it is not inhibited by 300 nM YVAD-aldehyde, a potent caspase-1 inhibitor ($IC_{50} = 1$ nM). Moreover, caspase-1 catalyzed caspase activation is also detected in immunoblots (Figure 1, panel B) using an antibody specific for the processed large subunit of several members of the caspase family, including caspase-3 (*31*). Processed caspase subunit is not detected in untreated heavy membrane samples (lane 1). However, a 20-kDa immunoreactive band corresponding to the large subunit of activated caspase is readily detected in caspase-1 treated samples (lane 4). The generation of the 20-kDa band was also observed in identical immunoblots probed using CSP3, a second caspase-3 specific antibody (unpublished data). This antibody is exclusively specific for caspase-3 (*21*), suggesting that the immunoreactive bands do indeed arise from caspase-3 activation. Pretreatment of caspase-1 with 300 nM YVAD-aldehyde inhibitor blocks both the activation of DEVD-AMC hydrolytic activity (Figure 1, panel A) and the generation of the 20-kDa large subunit (Figure 1, panel B, lane 3). At this concentration, YVAD-aldehyde completely inhibits caspase-1 but not caspase-3 ($IC_{50} > 10$ μM). These results indicate that caspase-1 enzymatic activity is required for membrane procaspase activation in these experiments.

The apparent molecular weight of the large subunit (20 kDa) of caspase-3 is similar to that of processed caspase-3 in apoptotic Jurkat T cells (*32*), yet it is significantly larger than the large subunit of purified bacterially expressed caspase-3 (lane 5). Unlike the bacterially expressed enzyme, the activated membrane caspase probably retains a partial or complete prodomain sequence on its N-terminus (*22*, *33*), since a fully intact C-terminus of the large subunit is required for immunoreactivity with this antibody (unpublished data).

Extraction of Native Procaspase from Heavy Membranes with Detergents. The procaspase is tightly associated with

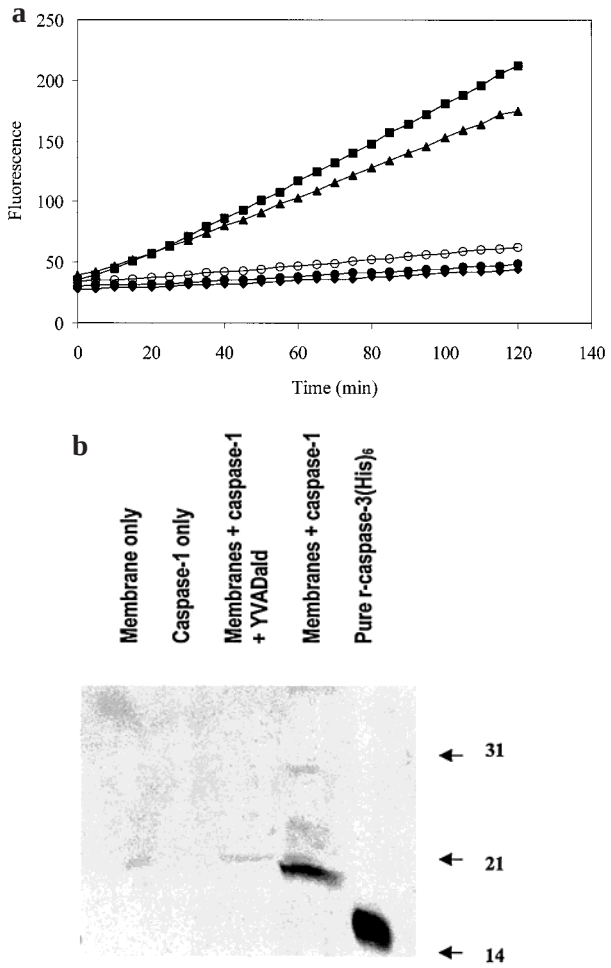

FIGURE 1: Activation of procaspase-3 associated with heavy membranes from 697 cells by caspase-1. (A) Activation of DEVD-AMC cleavage activity. Heavy membrane samples (65 μg of total protein/sample) from 697 cells were treated with recombinant murine caspase-1 (squares) or buffer (diamonds) for 1 h at room temperature (total volume of 60 μL). After centrifugation, 50 μL of each supernatant was added to 200 μL of 25 μM DEVD-AMC substrate in 96-well cytoplates; substrate cleavage in each well was monitored by the associated fluorescence increase as described in Materials and Methods . Caspase-1 activation can be blocked by the addition of 300 nM YVAD-aldehyde just prior to the 1 h incubation step (filled circles). Caspase-1-catalyzed DEVD-AMC hydrolysis (open circles) makes only a minor contribution to the observed hydrolysis activity and can be removed by adding 300 nM YVAD-aldehyde after the 1 h incubation step (triangles). (B) Immunoblot visualization of membrane procaspase processing. Supernatants from 697 heavy membrane samples were produced as described above and analyzed by SDS−PAGE. After electrophoresis, the proteins were transferred to PVDF membrane and immunoblotted using an antibody that recognizes the large subunit of caspase-3 as described in Materials and Methods. The positions of the molecular weight markers (kDa) are indicated to the right.

membranes; the protein/membrane interaction can withstand extensive buffer washes and freeze/thaw cycles (unpublished data). We decided to measure the effects of detergent on the membrane procaspase using two nonionic detergents, Triton X-100 and β-octyl glucoside. The membrane fractions were treated with detergents for 30 min on ice and then the insoluble pellet was removed by centrifugation. The localization of the procaspase was determined by treating the supernatant and pellet fractions with caspase-1 and measuring the DEVD-AMC hydrolysis activity in each fraction (Figure 2). Treatment of the membranes with either 0.1% Triton

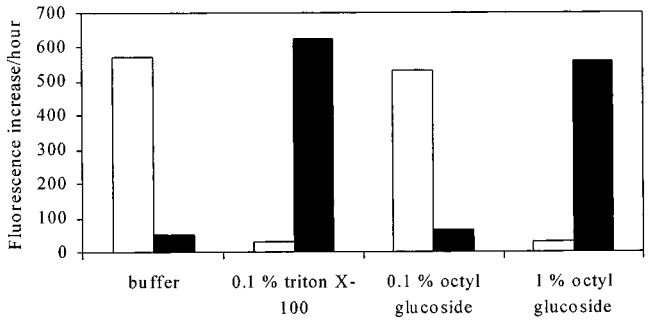

FIGURE 2: Procaspase-3 is tightly associated to heavy membranes and can be extracted with detergents. Washed 697 Neo heavy membranes were treated with one-tenth volume buffer or detergent (10× stock of final reaction concentration shown), incubated for 30 min on ice, and then centrifuged for 10 min at 14000*g*, 4 °C. The supernatant was removed, and the pellet was resuspended in an identical volume of hypotonic buffer. The resuspended pellet (open bars) and supernatant (shaded bars) samples were then activated with caspase-1 for 1 h at room temperature. The caspase-1 was then inhibited by the addition of one-tenth volume of 5 μM YVAD-aldehyde and the samples were centrifuged again. The DEVD-AMC cleavage activity of each of the supernatants was determined by diluting 50 μL of each supernatant into cytoplate wells containing 200 μL of 25 μM DEVD-AMC substrate and measuring the fluorescence increase in 1 h.

X-100 or 1% β-octyl glucoside efficiently extracted the procaspase from the membrane as indicated by the presence of caspase-1 activatable DEVD-AMC cleavage activity in the supernatant but not the membrane pellet. As expected, caspase-1-activatable DEVD-AMC cleavage activity remained associated with the membrane pellet in control buffer treated samples. Caspase-1 treatment is necessary for activation of the detergent-solubilized procaspase since no significant DEVD-AMC cleavage activity was detected in samples not treated with caspase-1 (unpublished data). This result indicates that solubilized procaspase does not spontaneously activate. Procaspase could not be extracted from the membranes when they were treated with 0.1% β-octyl glucoside (Figure 2), a concentration below the critical micelle concentration (CMC) for this detergent (0.7%). This result suggests that detergent micelle formation is required for procaspase solubilization. Very similar results were observed for CHAPS, a zwitterionic detergent with a CMC similar to that of β-octyl glucoside (unpublished data).

Affinity Purification of Activated Caspase from Membranes. To further identify and characterize the heavy membrane caspase, we purified the activated caspase using affinity chromatography (*9*). A 100-L culture of 697 cells was grown (110 billion cells total). Heavy membranes (193 mg total protein) were prepared from these cells as described in Materials and Methods. After caspase-1-mediated activation and solubilization of the heavy membrane caspase, it was purified using a column containing the DEVD-aldehyde affinity reagent bound to streptavidin-agarose as described in Materials and Methods. The activation of the procaspase with recombinant caspase-1 is necessary prior to purification since procaspase-3 does not bind the DEVD-aldehyde affinity reagent effectively. The heavy membrane caspase was eluted from the affinity column using two different elution conditions, denaturing and nondenaturing. SDS−PAGE analysis of the denatured affinity purified caspase indicates that the heavy membrane caspase is composed of a large 19-kDa subunit and a smaller 12-kDa subunit (Figure 3, panel A).

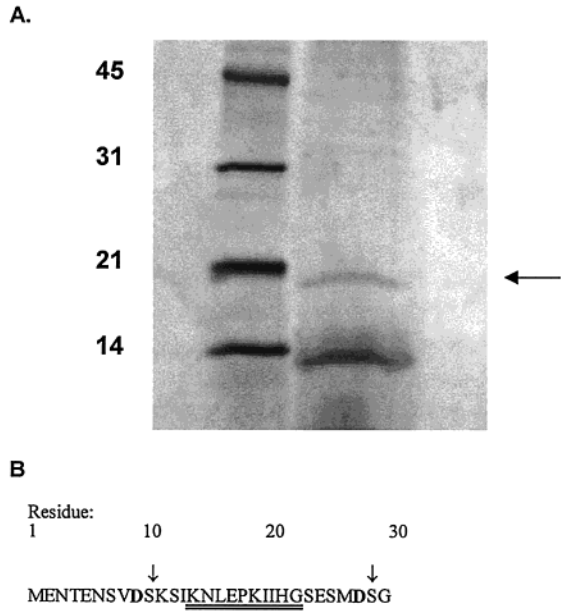

FIGURE 3: Affinity-purified heavy membrane caspase-3. (A) 15% SDS−PAGE analysis of affinity-purified membrane caspase from 697 cells. The protein bands were visualized with Coomassie stain. The position of the 19-kDa large subunit is indicated by the arrow. The molecular masses (kDa) of each of the molecular weight markers (lane 1) are indicated. (B) N-terminal sequence of human caspase-3 protein. The amino acid sequence of residues 14−23 of caspase-3 are identical to the observed N-terminal sequence of the 19-kDa band of the membrane-associated caspase (underlined). The procaspase-3 aspartic acid cleavage sites are in boldface type. The N-terminal residues of 697 cell cytoplasmic and recombinant bacterially expressed caspase-3 (Ser 10 and Ser 29, respectively) are indicated with arrows.

The amino acid sequence of the 19-kDa and 12-kDa bands were analyzed by N-terminal microsequencing (Figure 3, panel B). The N-terminal sequence of the first 10 residues of the 19-kDa subunit is identical to the sequence of residues 14−23 of human caspase-3, confirming the identity of the enzyme. The sequence of the 12-kDa band could not be determined because this band contained significant amounts of streptavidin that leached from the column during elution. The presence of streptavidin probably accounts for the disproportionately strong intensity of the 12-kDa band in the stained gel of the affinity-purified enzyme.

The apparent cleavage of the N-terminus of the enzyme after Ile 13 was unexpected since it has been demonstrated that procaspase-3 is processed by cleavage after the residues Asp 9 and Asp 28 in other cell systems (refs *9* and *22* and unpublished results). Our own results are in accord with these observations; the N-termini of the large subunits of caspase-3 from 697 cytoplasm (activated with caspase-1) and bacterially expressed recombinant caspase-3 are Ser 10 and Ser 29, respectively (Figure 3, panel B).

Enzymological Characterization of Purified Membrane Caspase-3. The heavy membrane caspase was also eluted from the affinity column under nondenaturing conditions using hydroxylamine/glutathione as described (*8*). The oxidized caspase was rapidly reduced by 6 mM DTT to its active form, which efficiently cleaves the DEVD-AMC substrate (Figure 4). The steady-state kinetic and inhibitory parameters of the reactivated caspase were measured to compare the heavy membrane caspase-3 to previously isolated forms of caspase-3 from other sources. The observed

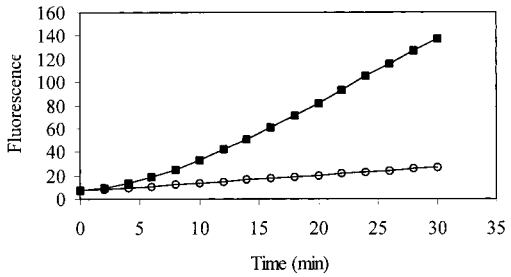

FIGURE 4: DEVD-AMC hydrolysis activity of affinity purified caspase-3 from 697 cell membranes. Desalted affinity-purified heavy membrane caspase-3 was diluted into 20 μM DEVD-AMC substrate solution. The enzyme is rapidly activated in substrate containing 6 mM DTT (squares), while little or no activation occurs in the absence of DTT (circles).

Table 1: Comparison of Caspase Inhibitor IC_{50} Values of Purified Membrane and Recombinant Caspase-3 Proteins

	IC50 (nM)	
inhibitor	membrane	r-caspase-3 (His)6
DEVDalde	1.0	1.0
ZEVDalde	32	24
DFLDalde	4.7	1.1
VEIDalde	34	32
baculovirus p35	0.074	0.041

K_M value for the purified caspase (10 μM) is essentially identical to the K_M value for recombinant Ni^{2+}-purified caspase-3 as well as caspase-3 isolated from THP-1 cell cytoplasm (*9*). The inhibition of DEVD-AMC cleavage activity by several caspase inhibitors were also measured (Table 1). For each peptide aldehyde, the IC_{50} values for the inhibition of membrane caspase-3 are very similar to those of recombinant caspase-3. The heavy membrane caspase from 697 cells is also potently inhibited by recombinant baculovirus p35 protein (IC_{50} = 74 pM). Using this IC_{50} to set an upper limit on the enzyme concentration, the k_{cat}/K_M for DEVD-AMC hydrolysis must be $\geq$400 000 M^{-1} s^{-1}, which is close to the k_{cat}/K_M value for the recombinant caspase-3 (1 200 000 M^{-1} s^{-1}). Collectively, our data indicate that affinity purified membrane-associated caspase-3 and Ni^{2+}-purified recombinant caspase-3 are functionally indistinguishable.

DISCUSSION

In this study, we have described the identification and purification of caspase-3 from 697 cell heavy membrane fractions. Although activated caspase-3 has been purified from various cytoplasmic extracts (*9*, *22*, *23*) and the procaspase has been immunocytochemically identified in noncytoplasmic compartments such as mitochondria (*29*), this report describes for the first time the purification and characterization of caspase-3 that is associated to cell heavy membranes.

Mancini et al. (*29*) have demonstrated that procaspase-3 is distributed both in cytoplasm and in the intermembrane space of mitochondria. Since heavy membrane (HM) fractions contain mitochondria (*21*), one must not overlook a slim possibility that the HM-associated caspase-3 characterized in this paper was from the caspase-3 located within mitochondria. Several observations have led us to rule out this possibility. First, it was demonstrated (*21*) that this HM-

associated procaspase-3 migrates at a slower rate than cytoplasmic procaspase-3 on the SDS electrophoresis gel, whereas the procaspase-3 from intermembrane space of mitochondria comigrates with the cytoplasmic procaspase-3 in an indistinguishable manner (*29*). Although the nature of the slower movement of HM-procaspase-3 on the SDS gel is not completely understood, it does provide a clear distinction that stands out the HM-procaspase-3 from cytoplasmic procaspase-3 and the mitochondrial procaspase-3 reported by Mancini and co-workers. Second, it was very clearly demonstrated by Mancini et al. that the caspase 3 they reported was not a membrane protein but was indeed localized to the mitochondria intermembrane space. On the other hand, the HM-caspase-3 we report here is of little doubt a membrane-associated protein before activation. This is exemplified by the need for detergents at concentrations above CMC to disengage the tight association of the procaspase with heavy membranes (Figure 2). At detergent concentrations below CMC but sufficient for the permeation of cytochrome C out of inner mitochondria exhibited no effect on releasing of the membrane-associated procaspase-3. The observation that constant freeze-and-thaw and extensive washes of the membrane resulted in no detectable loss of the procaspase is consistent with the membrane-philic nature of the HM-procaspase-3, since these manipulations are known to severely compromise the integrity of intact mitochondria and cause the components in the intermembrane space to be released from the mitochondria. Third, unlike the inner mitochondrial procaspase-3 described by Mancini et al. (*29*), our membrane procaspase-3 can be proteolytically activated either by caspase-1 (Figure 1) or by granzyme B (*21*) in the absence of detergents. Since caspase-1 and granzyme B are unlikely to translocate across mitochondrial membranes, it is most likely that the HM-procaspase-3 is located at the outer membrane surface to allow such cleavages/activation. Could it be possible that the procaspase-3 activated with this procedure is derived from mitochondrial leakage? We have observed that the activation of HM-procaspase-3 begins immediately after caspase-1 addition to membranes and that the rate of activation is unaffected by the overexpression of Bcl-2 (unpublished data). Given that Bcl-2 can block the release of proteins (such as cytochrome *c*) from mitochondrial intramembrane space (*34*, *35*), it is very difficult to reconcile that mitochondrial leakage played any role in the observed activation.

We have used immunocytochemical techniques to probe the subcellular distribution of caspase-3 in 697 and other cell types. While these studies clearly indicate that caspase-3 is associated with mitochondria (*21*), the resolution of immunocytochemical imaging was insufficient to allow the determination of its precise membrane topology.

In addition to the observation of this caspase activity in both heavy membranes and purified mitochondrial fractions (*21*), a membrane-associated procaspase similar to the one we report here is also present in the nuclear fractions (devoid of mitochondria) from 697 cells. Biochemical and immunological characterization suggested that it is also a caspase-3 (unpublished data). Thus, heavy membranes may not be the only membrane that procaspase-3 associates to; indeed, it is also associated to the nuclear membranes. Collectively, our data indicate that a portion of cellular procaspase-3 is localized to membranes. Although it is unclear how procaspase-3 is physically associated with heavy membranes, the requirement for detergent micelles for solubilization (Figure 2) suggests that procaspase may be integrally bound to the membrane.

Like HM-procaspase-3, Bcl-2 is localized to the heavy membrane fraction (as well as the nuclear fraction) of 697 cells. It may not be coincidental that noncytoplasmic procaspase-3 and Bcl-2 are colocalized within the same compartment; the sequestration of procaspase-3 to membranes may allow the direct suppression of procaspase activation by Bcl-2. While it has been demonstrated that Bcl-2 can block procaspase-3 activation by preventing the release of cytochrome *c* from mitochondria (*34*, *35*), Bcl-2 has also been shown to block procaspase-3 activation after cytochrome *c* has been released into the cytoplasm (*36*). Our observation that procaspase-3 is sequestered to Bcl-2-containing membranes suggests that, in addition to blocking the release of cytochrome *c*, Bcl-2 may also be repressing the activation of procaspase-3 by blocking the activation of membrane bound procaspase. Since Bcl-2 overexpression does not affect the amount of procaspase-3 bound to heavy membranes, Bcl-2 does not merely block the activation of procaspase-3 by sequestering it from the cytoplasm but rather by blocking the activation of procaspase-3 already bound to the membrane (*21*). The suppression of activation caused by Bcl-2 can be circumvented by caspase-1 treatment. Once activated, caspase-3 dissociates from the membranes. This dissociation is not inhibited by the overexpression of Bcl-2, suggesting that activated procaspase-3 does not appreciably bind to the Bcl-2 protein (or Bcl-2/caspase adapter molecules). The mechanism by which activation triggers dissociation is not known. However, our studies suggest that release of activated caspase-3 from the membrane may be caused by a conformational change in the caspase-3 protein that disrupts the membrane complex or inactivation of proteins within a putative heavy membrane complex by caspase-mediated proteolysis.

It is generally accepted that caspase-3 activation is caused by cleavage of the zymogen after the Asp 175 residue, which causes the liberation of the mature small subunit (*18*, *37*). In our studies, activation of membrane procaspase-3 by caspase-1 may also occur by the same mechanism since the Ile 172−Asp 175 site seems to be the most similar to the canonical caspase-1 cleavage site identified by substrate specificity studies (*38*). Also, the tetrapeptide inhibitor derived from the Asp 175 cleavage site, IETD-aldehyde is a highly potent caspase-1 inhibitor ($IC_{50} = 11$ nM), suggesting that caspase-1 possesses a high affinity for this sequence. After activation by cleavage at Asp 175, caspase-3 might autocatalytically catalyze subsequent cleavage at the Asp 9 and/or Asp 28 residues in the prodomain/large subunit junction (*18*, *37*).

Previous studies of membrane-associated procaspase-3 indicate that the activation of the enzyme precedes its release from the membrane (*21*). Nevertheless, it has been difficult to formally ascertain whether caspase activation precedes release or vice versa. Our observation that detergent-solubilized membrane procaspase-3 is not catalytically active suggests that membrane association itself does not inhibit activation and supports the hypothesis that procaspase activation precedes membrane release.

We have used affinity chromatography to purify active caspase-3 from 697 cell membranes in fully active form. Nicholson et al. (*9*) and Faleiro et al. (*22*) used a similar strategy to purify caspase-3 from activated THP-1 and Jurkat cell cytoplasm, respectively. The N-terminal residue of the large subunit of caspase-3 purified from these cytoplasmic extracts is Ser 29. Conversely, our studies indicate that the N-terminal residues of the large subunit of activated caspase-3 purified from the cytoplasm and membranes of 697 cells are Ser 10 and Lys 14, respectively (Figure 3). This latter result is particularly interesting since it is generally accepted that caspase activation occurs via cleavage after aspartic acid residues; the closest such residue is Asp 9. Perhaps the large subunit was cleaved after the Asp 9 residue and then residues 10−13 were removed by a second protease. Alternatively, caspase-3 might be specifically cleaved after Ile 13 by a novel membrane-associated protease. It is however also possible that a portion of caspase-3 zymogen was cleaved at Ile 13 by a cytoplasmic protease and consequently resulted in a conformational change of the zymogen that subsequently dissociated from the heavy membranes. Although two additional residues, Ser 10 and Lys 11, were removed from the N-terminus of hamster caspase-3 isolated from liver S-100 supernatant (*23*), these residues were not removed from caspase-3 isolated from 697 cytoplasm in our studies (Figure 3).

We have characterized the enzymological properties of the affinity-purified caspase and compared them to those of purified recombinant enzyme. The values for the steady-state kinetic parameters (k_{cat}/K_M and K_M) for the hydrolysis of tetrapeptide substrates are very similar for the affinity-purified and Ni^{2+}-purified enzymes. Similarly the IC_{50} values for tetrapeptide aldehyde inhibitors are very similar between the two forms of caspase-3 (Table 1) as well as the IC50 values for caspase-3 in 697 cell cytoplasmic extracts (unpublished data). This result is interesting since the N-terminal residues of the large subunits of these proteins are very different (Figure 3). Our results clearly indicate that residues in this alternatively processed span, Ser 10−Asp 28 play no significant role in catalysis or inhibitor binding; the procaspase can be cleaved either at or near Asp 9 or Asp 28 with no detrimental consequences to catalytic function. This span may reside far from the active site since the crystal structures of recombinant caspase-3 indicates that the observed N-terminus (Ser 36) is quite remote from the active sites of the enzyme (>25 Å). However, all preceding residues are too disordered to be observed (*5*, *6*), and therefore it is difficult to be absolutely certain that this span has no interactions with the active site of the enzyme.

Since there is no detectable functional consequence of the differential processing of the N-terminus of the large subunit, what is the significance of the alternative processing sites? Perhaps these residues play a role in the activation of the procaspase or allow the mature enzyme to interact with intracellular factors. If so, these regulatory residues might play an important role in mediating interactions with factors for specific types of apoptotic stimuli or subcellular compartments. Interestingly, Erhardt and Cooper (*39*) found that the processing of the large subunit of caspase-3 in U937 cells was dependent on the apoptotic stimuli utilized. The ability of the Bcl-2 homologue Bcl-x_L to inhibit apoptosis from various stimuli was correlated with the extent of processing of the caspase-3 large subunit (putatively at Asp 28). Perhaps these residues near the N-terminus of the protein play an important regulatory role that controls the localization and specific activation of procaspase-3. Currently, we are performing additional studies to understand the molecular mechanism of procaspase-3 processing and its association to membranes. These studies will help us to better understand the specific mechanisms of caspase activation and its regulation by membrane proteins such as Bcl-2.

ACKNOWLEDGMENT

We thank Drs. H.R. Horvitz, D. Xue, J. Reed, and E. Alnemri for cDNA clones and cell lines, Lyrelene Fernandez and Mubina Moledina for technical assistance, and Lisa Trout for invaluable assistance in the preparation of this manuscript.

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BI001007W