Lysosomal Protease Pathways to Apoptosis

CLEAVAGE OF Bid, NOT PRO-CASPASES, IS THE MOST LIKELY ROUTE*

We investigated the mechanism of lysosome-mediated cell death using purified recombinant pro-apoptotic proteins, and cell-free extracts from the human neuronal progenitor cell line NT2. Potential effectors were either isolated lysosomes or purified lysosomal proteases. Purified lysosomal cathepsins B, H, K, L, S, and X or an extract of mouse lysosomes did not directly activate either recombinant caspase zymogens or caspase zymogens present in an NT2 cytosolic extract to any significant extent. In contrast, a cathepsin L-related protease from the protozoan parasite Trypanosoma cruzi, cruzipain, showed a measurable caspase activation rate. This demonstrated that members of the papain family can directly activate caspasess but that mammalian lysosomal members of this family may have been negatively selected for caspase activation to prevent inappropriate induction of apoptosis. Given the lack of evidence for a direct role in caspase activation by lysosomal proteases, we hypothesized that an indirect mode of caspase activation may involve the Bcl-2 family member Bid. In support of this, Bid was cleaved in the presence of lysosomal extracts, at a site six residues downstream from that seen for pathways involving caspase 8. Incubation of mitochondria with Bid that had been cleaved by lysosomal extracts resulted in cytochrome c release. Thus, cleavage of Bid may represent a mechanism by which proteases that have leaked from the lysosomes can precipitate cytochrome c release and subsequent caspase activation. This is supported by the finding that cystolic extracts from mice ablated in the bid gene are impaired in the ability to release cytochrome c in response to lysosome extracts. Together these data suggest that Bid represents a sensor that allows cells to initiate apoptosis in response to widespread adventitious proteolysis.

In mammals, programmed cell death can be initiated by three distinct pathways: (i) the extrinsic pathway, which can be triggered by ligation of death receptors and subsequent caspase 8 activation; (ii) the intrinsic pathway, which is initiated by cellular stress followed by activation of caspase 9; or (iii) the granzyme B pathway, where the cytotoxic cell protease granzyme B is delivered to sensitive target cells. Each of these pathways converges to a common execution phase of apoptosis that requires the activation of caspases 3 and 7 from their inactive zymogen form to their processed, active form (1, 2). The apical activators, caspase 8 and 9, and granzyme B all have a primary specificity for cleavage at Asp297 (caspase 1 numbering convention), located in a region that delineates the large and small subunits of active caspases 3 and 7.

The activation of the cell death pathway depends on both the triggering stimulus and the cell type (3), and in many forms of apoptosis cytochrome c release from mitochondria is important for activation of downstream caspases (4). The Bcl-2 protein family contains both pro- and anti-apoptotic members that can act as an upstream checkpoint of caspase activation at the level of the mitochondria by controlling cytochrome c release. Bid, a pro-apoptotic member of the family, has recently been identified as a target for proteolytic cleavage by caspase 8 and granzyme B (5–8). Activated caspase 8 cleaves Bid at Asp297 to trigger translocation from the cytosol to the mitochondria where it promotes cytochrome c release.

Direct cleavage of both Bid and the downstream caspases can promote death pathways; however, it is unclear to what degree specificity of cleavage is required. For example, whereas processing of the caspase 3 and 7 zymogens at Asp297 is considered to be the dominant physiologic pathway for activation, cleavage of pro-caspase 7 at Gln285 is sufficient to activate the zymogen in vitro (9). Such results suggest that alternative proteolytic events may be sufficient to activate pro-caspases and perhaps Bid cleavage, especially in pathologic instances where proteolysis tends to be unregulated.

The lysosome is the primary reservoir of nonspecific proteases in the mammalian cell. In certain pathological situations, as well as during normal aging (10, 11), lysosomal integrity may be compromised, causing leakage of lysosomal proteases into the cytosol. Thus, certain diseases related to lysosomal pathology may have a primarily apoptotic component. Several lines of evidence support this possibility: (i) leakage of lysosomal proteases into the cytosolic compartment may be involved in the activation of caspases (12); (ii) in Jurkat...
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T-cells, α-tocopheryl succinate triggers apoptosis and caspase 3 activation accompanied by lysosomal destabilization (13); and (iii) the lysosomal protease cathepsin B has been implicated in the activation of the proinflammatory caspases 11 (14) and 15 (15) as well as in the induction of the nuclear morphology associated with apoptotic cells (15).

The role of lysosomal proteases in the activation of the apoptotic pathway is unclear. To examine the possibility that they may be involved in programmed cell death, the activity of both recombinant cathepsins and lysosomal extracts on recombinant caspases and cysteine extracts was examined. We tested two hypotheses: that lysosomal proteases may directly activate executioner caspases and that lysosomal protease cleavage of Bid may separately trigger the intrinsic apoptosis pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphate-buffered saline, fetal bovine serum, cytochrome c, Percoll, EGTa, p-i-donitrotetrazolium violet, penicillin, streptomycin, anti-mouse and anti-rabbit horseradish peroxidase antibodies, and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside were purchased from Sigma. All other chemicals were of analytical grade. Human cathepsins B (16), H (17), K, (18), L (19) S (20), and X (21), cruzipain (22), and human granzyme B (23) were purified as described previously (25–28), and human low molecular weight subtilisin BPN′ (21), cruzipain (22), and human granzyme B (23) were purified as cited. Human stefins A and B (24) and human cystatins C, D, E/M, and F were purified as described previously (25–28), and human low molecular weight subtilisin BPN′ (21), cruzipain (22), and human granzyme B (23) were purified as cited.

**Human Liver Isolation**—Liver tissue was obtained from patients undergoing orthotopic liver transplantation and was immediately placed on ice. Liver tissue was cut into 1 cm3 cubes and homogenized by two brief pulses from a Brinkman Polytron homogenizer. The homogenate was centrifuged for 10 min at 540g for 10 min to pellet the lysosomal membranes, and the supernatant was assayed. The enzyme released product was measured continuously during 20 min at 37°C using the fluo-3 fluorescence microplate reader, at excitation and emission wavelengths of 355 and 460 nm, respectively.

**Mitochondria**—A 25-μl sample of succinic p-i-donitrotetrazolium reductase was incubated with 75 μl of 2 mM p-i-donitrotetrazolium violet in 55 mM potassium dihydrogen phosphate and 55 mM succinic acid, pH 6.0, containing 250 mM sucrose until a pink color caused by product formation developed. The reaction was stopped by adding 125 μl of 10% trichloroacetic acid, and the pellet was resuspended in 100 μl of ethanol. The sample was clarified at 16,000 × g for 5 min, and the absorbance of the supernatant was read at 495 nm on a SpectraMAX 340 spectrophotometric plate reader (Molecular Devices).

**Isolation of Mouse Lysosomes**—Lysosomes were purified from mouse liver as described previously with several modifications (36, 38). All steps were carried out at 4°C unless otherwise noted. Briefly, several livers were washed with sucrose/Pipes buffer (250 mM sucrose, 20 mM Pipes, pH 7.2), resuspended in 10 volumes sucrose/Pipes buffer and homogenized by two brief pulses from a Brinkman Polytron homogenizer. The homogenate was centrifuged for 10 min at 540g for 10 min to remove nucleic and particulates. CaCl2 (final concentration, 1 mM) was added to the supernatant, followed by incubation for 5 min at 37°C to disrupt the mitochondria. The supernatant was centrifuged for 10 min at 18,000 × g, and the heavy membrane pellet was retained. At this point, the integrity of the lysosomes was verified by comparing the activity of the lysosomal and the pellet with the lysosomal protease substrate Z-FR-AMC in the presence and absence of a lysosomotropic detergent (Triton X-100; final concentration, 1% v/v). If the lysosomes were judged at least 80% intact, the heavy membrane fraction was resuspended in sucrose/Pipes buffer, centrifuged again for 10 min at 15,000 × g, and resuspended in Percoll (40% w/v) in sucrose/Pipes. The Percoll solution was centrifuged for 30 min at 44,000 × g to form a gradient, and 1-ml fractions were collected from the bottom and assayed for mitochondrial contamination using the lysosomal and mitochondrial enzyme markers as described above. The lysosomal fractions were pooled, diluted in sucrose/Pipes (1:10 v/v) to decrease the Percoll, and pelleted by centrifugation at 17,000 × g for 10 min. The lysosomal pellet was washed, resuspended in an equal volume of sucrose/Pipes, and stored at −70°C.

**Soluble lysosomal constituents were released by three freeze-thaw cycles with a 15-s vortex between each cycle. The suspension was centrifuged at 10,000 × g for 10 min to pellet the lysosomal membranes, and the supernatant was saved.”**

**Isolation of Mitochondria**—Mitochondria were isolated from rat heart according to the protocol described in the procedure described in Ref. 39. Protein content was determined by Bradford assay (Bio-Rad), and the mitochondria were stored on ice at 3 mg/ml mitochondrial protein. The mitochondrial were used within 2 h of preparation.

**Cell-free Extract Preparation and Detection of Endogenous Caspase Levels**—A cytosolic extract from the human neuronal cell line NT2/D1 was prepared as described previously (40). Protein concentration was determined using the Bradford assay (Bio-Rad), and the extract was diluted to 1 mg/ml by the addition of potential caspase activators (cytochrome c or proteases). Samples were run on SDS-PAGE on 8–18% gradient acrylamide gels, electrorephoretically transferred to Immobilon-P membrane (Millipore), and probed with antibodies against human caspases. Caspase-antibody complexes were detected with horseradish peroxidase conjugated goat anti-rabbit IgG using ECL (Amersham Pharmacia Biotech).

**Caspase Activation**—The activation of caspasezymogens, whether recombinant or in NT2 cytosolic extracts, was followed fluorometrically by monitoring AFC release from the substrate Ac-DEVD-AFC. A 5-μl aliquot of NT2 cytotoxic extract (14 mg/ml) or zymogens of caspases 3 and 7 (70 μM) were incubated in assay buffer (50 mM Hepes, 100 mM NaCl, 0.1% (w/v) CHAPS, 10 mM DTT, pH 7.4) with potential proteolytic activators in the range of 1 nM to 1 μM at 37°C for 30 min, (total volume, 50 μl). For analysis of potential activators in the lysosomal extract, 2–5 μl of extract was used in place of the purified proteases. Activation was measured by adding 50 μl of the substrate Ac-DEVD-AFC (200 μM) in 96-well microplate format to a 37°C thermostatted fluorescence microplate reader (Molecular Devices), and caspase activity was determined at excitation and emission wavelengths of 380 and 505 nm, respectively. The concentration of substrate was determined continuously for 30 min. The steady-state hydrolysis rates were obtained from the linear part of the curves.

The instantaneous rates of cisuprain-mediated activation for caspasezymogens 3 and 7 were determined as described previously (9). Briefly, caspase zymogen 3 (final concentration, 92.3 μM) or caspase zymogen 7 (final concentration, 20 μM) were added to the substrate, the reaction

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1 The abbreviations used are: Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-7-p-nitroanilide; Ac-DEVD-AFC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Z-FR-AMC, benzoylcarbonyl-Phe-Arg-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) were obtained from Bachem and Enzyme Systems Products, respectively. The inhibitors E-64 and benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were from Pep tide Research Institute and Enzyme Systems Products, respectively. Stock solutions of the substrates and inhibitors were prepared in dimethyl sulfoxide and stored at −20°C for up to 12 months.

**Biochemical Markers for Lysosomes and Mitochondria**—Because the purity of lysosomes and mitochondria was paramount in this study, we analyzed our preparations with one mitochondrial and two lysosomal markers to minimize any cross-contamination between these organelles. For lysosomes we followed the activity of lysosomal proteases on Z-FR-AMC substrate (35) and β-hexaminidase using a method modified from (36). For mitochondria, we used succinic p-i-donitrotetrazolium reductase (37). Test samples during the purifications were treated with Triton X-100 (final concentration, 1% w/v) and spun at 9,800 × g for 5 min, and the supernatant was assayed. The enzyme assays were carried out at 37°C. Controls in the absence of the sample were run under the same conditions.

**Lysosomes**—A 5-μl sample of each fraction was added to a 96-well plate. The reaction started by addition of 75 μl of warmed 10 μM Z-FR-AMC in 100 mM phosphate buffer, pH 6.0, containing 1 mM DTT and 250 mM sucrose. The released product was measured continuously during 5 min at 37°C using a fura-3 fluorescence microplate reader (Molecular Devices), at excitation and emission wavelengths of 355 and 460 nm, respectively. For β-hexaminidase assays, a 25-μl sample was added to a 96-well plate. The reaction was started by addition of 75 μl of 2 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 400 mM acetate buffer, pH 4.4, containing 250 mM sucrose. The released product was measured continuously during 20 min at 37°C using the fura-3 fluorescence microplate reader, at excitation and emission wavelengths of 355 and 460 nm, respectively.
was started by cruzipain addition to final concentrations of 50, 200, or 300 nM, and the time course was followed continuously for 30 min. In separate experiments to characterize the NT2 cytosolic extracts, granzyme B (final concentration, 20 nM) or cytochrome c (final concentration, 10 nM) was added, and processed forms of the extract were measured spectrophotometrically (30 min at 37 °C). Although the NT2 extracts usually activated equally well in the presence or absence of dATP, we kept this as a standard procedure (41), although subsequent descriptions may refer to cytochrome c alone. The release of p-nitroanilide was continuously recorded during 30 min at 405 nm in 96-well microplate format using a SpectraMax 340 spectrophotometric plate reader (Molecular Devices) thermostatted at 37 °C.

Inhibition Studies—The active concentration of each of the purified lysosomal proteases was standardized by using E-64 (42), and the active concentration of caspases was standardized by a similar protocol utilizing Z-VD-Fmk (30). The endogenous anti-lysosomal protease concentration in NT2 cytosolic extracts was determined by titration using standardized proteases where 5 μl of each enzyme (0.1–2 nM) was added to 40 μl of extract in the presence of Ac-DEVD-pNA (final concentration, 100 μM). Although the NT2 extracts usually activated equally well in the presence or absence of dATP, we kept this as a standard procedure (41), although subsequent descriptions may refer to cytochrome c alone. The release of p-nitroanilide was continuously recorded during 30 min at 405 nm in 96-well microplate format using a SpectraMax 340 spectrophotometric plate reader (Molecular Devices) thermostatted at 37 °C.

Isolation of Liver Mitochondria and Cytosol from Wild Type and bid-deficient Mice—The procedure is essentially conducted as described previously (5). Livers of wild type and bid-deficient mice (8) were homogenized with a Dounce homogenizer in CRM medium containing 25 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM MgCl2, 2 mM EDTA. The homogenate was centrifuged at 10,000 × g at 4 °C for 20 min to precipitate the heavy membrane fractions (mitochondria). The mitochondrial pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl with 5 μM of SDS sample buffer and heated at 100 °C for 5 min. 5 μl of sample buffer was added to the supernatant fractions. The samples were resolved on a 15% Hi-Tris gel (43). The gel was transferred to nitrocellulose (Schleicher & Schuell) and probed with anti-cytochrome c antibody. Antibody complexes were detected with a horseradish peroxidase-conjugated goat-anti mouse IgG (Bio-Rad) using ECL (Amersham Pharmacia Biotech). Several exposures were taken for each blot. Total cytochrome c content is represented by mitochondrial samples treated with 1% Triton X-100.

Isolation of Liver Mitochondria and Cytosol from Wild Type and bid-deficient Mice—The procedure is essentially conducted as described previously (5). Livers of wild type and bid-deficient mice (8) were homogenized with a Dounce homogenizer in CRM medium containing 25 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM MgCl2, 2 mM EDTA. The homogenate was centrifuged at 1,000 × g for 10 min at 4 °C to remove intact cells and nuclei, and the supernatants were further centrifuged at 10,000 × g at 4 °C for 10 min to precipitate the heavy membrane fractions (mitochondria). The mitochondrial pellet was resuspended in the same buffer. Mitochondria were kept on ice and used within 2 h of preparation. The supernatants were further centrifuged at 100,000 × g for another 60 min to obtain the cytosolic extracts used in the assay.
RESULTS

Characterization of Human NT2/D1 Neuronal Precursor—The human NT2/D1 teratocarcinoma cell line, a neuronal progenitor, was chosen for generating cytosolic extracts because it responds well to a variety of apoptotic stimuli (40, 44). Caspases 2, 3, 6, 7, 8, 9, and 10, the human caspases currently considered to participate in apoptosis signaling, are present in the cytosolic extract (Fig. 1A). We were able to determine some of the caspase concentrations in the cytosolic extract by semiquantitative Western blot analysis, where standard recombinant caspases were compared with the endogenous NT2 amounts (for results see Fig. 1A). These degrees of activation were compared with that generated by the physiologic caspase activator granzyme B (GB, in the main panels of A and B, respectively). All experiments were performed in triplicate, and the standard deviations were less than 10% of the indicated values.

Caspase activation in extracts was followed after the addition of cytochrome c, which resulted in processing of pro-caspases 2, 3, 6, 7, 8, and 9 to their presumptive active forms, whereas the upstream caspases 8 and 10 were not processed under the same conditions (Fig. 1A). The lack of pro-caspase 8 or 10 processing is in contrast to previous results with Jurkat T-cell extracts (45) but consistent with the general conclusion that these initiators do not participate in executioner caspase activation by the intrinsic (mitochondrial) pathway. We offer no explanation for the processing of pro-caspase 2, because its function in apoptotic pathways is still debated (46, 47). Activation was also followed by observing the increase in activity against the substrate Ac-DEVD-pNA (Fig. 1B), which predominantly reflects activation of pro-caspase 3, although other caspases will also contribute to the increase in activity (48). This result is consistent with cytochrome c-mediated activation of pro-caspase 9, which then amplifies the cascade through the activation of downstream caspases 3, 6 and 7 (as reviewed in Refs. 2 and 4).
Caspase activation can also be achieved in extracts by adding the serine protease granzyme B, again demonstrated by an increase in the cleavage of Ac-DEVD-pNA (Fig. 1B). Both pathways showed the same efficiency and can be considered to represent the maximal caspase activity that could be achieved within the extract. All subsequent comparisons of caspase activation by lysosomal proteases were therefore compared with the physiologic activator granzyme B.

**Proteolytic Activation of Pro-caspases 3 and 7**—Because recombinant zymogens of caspases 3 and 7 are available (34), we were able to use these to determine whether lysosomal proteases might directly activate these executioner caspases. Pro-caspases were incubated with 1, 10, 100, or 1000 nM of the respective purified protease for 30 min at 37 °C, and the maximal caspase activities generated (in all cases at 1000 nM protease) are shown in Fig. 2. Because some lysosomal proteases are irreversibly inactivated at neutral pH but are stable in the slightly acidic range of around 6.0 (49), activations were also conducted at pH 6.0, a level optimal for the lysosomal proteases, and then raised to the normal cytosolic pH of 7.2 for assay of caspase activity. Duplicate assays were conducted with the activation period at pH 7.2. The data shown from this point were all collected with activation at pH 7.2, because activation at pH 6.0 produced no more caspase activity than at pH 7.2. Both cruzipain and cathepsin B cleaved Ac-DEVD-pNA, supporting previous observations of nonspecific reaction of lysosomal proteases with ostensibly specific caspase reagents based on similar sequences (50). These background values were subtracted before the data were compared.

Cathepsins B, H, K, L, S, and X were unable to activate caspase zymogens 3 and 7 directly. In contrast, cruzipain (the cathepsin L homolog from *Trypanosoma cruzi*) appeared to induce a substantial activation of pro-caspase 3 and pro-caspase 7 (Fig. 2, insets). However, when compared with the maximal activity obtained by granzyme B treatment, the cruzipain activation of caspase zymogens 3 and 7 was only 10 and 3%, respectively (Fig. 2, main panels). The activation of pro-caspase 3 was too slow to be measured, but we were able to determine the rate of activation of pro-caspase 3 by cruzipain to be $1.7 \times 10^3$ M$^{-1}$ s$^{-1}$, with activation following cleavage after Cys$^{170}$ within the sequence ELDC$^{170}$GIETD$^{172}$S, five residues upstream of the canonical zymogen activation site (Asp$^{175}$) within the caspase 3 zymogen. The slow activation rate is unlikely to be physiologically relevant, although it cannot be ruled out that the intracellular parasite form of *T. cruzi* may utilize cruzipain to directly activate caspases at some stage during its presence in infected host cells. Clearly, within the wide array of parameters used in this preliminary study, none of the purified human lysosomal cysteine proteases can be construed as direct activators of the execution caspases.

**Activation of NT2 Cytosolic Extract by Lysosomal Proteases**—To examine whether the purified lysosomal proteases could activate pro-caspases other than 3 and 7, we utilized NT2 cytosolic extracts as the source of caspase zymogens. Cathepsins B and X caused a slight increase in caspase activity (Fig. 3, inset). Cruzipain was significantly more efficient, exhibiting about a 2.5-fold increase in Ac-DEVD-pNA cleaving activity relative to background (Fig. 3, inset). Granzyme B induced maximal activation in NT2 cytosolic extracts, and although cruzipain was a better activator than the cathepsins, its activity was only 5% that of granzyme B (Fig. 3).

Because purified lysosomal enzymes showed only a minor activation of caspases in the NT2 cytosolic extract, a lysosomal extract was next used to explore whether additional proteases not included in our purified repertoire could cause caspase activation in NT2 extracts. The lysosomal extract possessed no detectable activity against Ac-DEVD-AFC and induced a 2-fold activation of the NT2 extract (Fig. 4, inset). However this is only 5% of the activation conducted by granzyme B (Fig. 3).

### Table I

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<th>Enzyme</th>
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<tr>
<td>Cathepsin B</td>
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<td>Cathepsin K</td>
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<td>Cathepsin L</td>
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<td>Cathepsin X</td>
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<tr>
<td>Cruzipain</td>
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**Inhibition of lysosomal cysteine proteinases by NT2 cytosolic extract**

Total inhibitory capacities in NT2 extracts against each of the purified, standardized lysosomal proteases are shown. NI, not inhibited.

### Table II

**Effect of cystatins on caspases**

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<th>Inhibitor</th>
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### Table III

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**Inhibition of Lysosomal Cysteine Proteases by the NT2 Cytosolic Extract**—One could argue that the lack of substantial activation of pro-caspases in the NT2 cytosolic extract by lysosomal proteases may be due to endogenous inhibitors. To explore this, we titrated the NT2 extract against standardized proteases, which revealed that cathepsins B, H, L, S, and X and cruzipain were inhibited in the range of 0.6–25 nM, whereas cathepsin K was not inhibited (Table I). Clearly the presence of endogenous caspase inhibitors can be ignored, because both granzyme B and cysteine activated the extracts very well. Nevertheless, one could argue that cystatins, being inhibitors of lysosomal cysteine proteases, may also inhibit caspases and, therefore, caspase activation. To check this we tested representatives of all three known families of cystatins for inhibition of caspases 3, 6, 7, and 8. No substantial inhibition was observed, even at vast excess of cystatins (Table II), so we can effectively rule cystatins out as caspase regulators.

**Cleavage of Bid by the Lysosomal Extract**—Because the caspase pathway in cytosolic extracts was not activated directly by lysosomal proteases, we explored whether lysosomal extracts could cleave protein intermediates to play a role in caspase activation. An obvious place to look for this is in the mitochondrial amplifier of the intrinsic apoptosis pathway. This pathway encompasses the participation of several members of the Bcl-2 family, of which Bid is an attractive candidate because it is activated through proteolytic cleavage by caspase 8 or granzyme B. Truncated Bid translocates to the mitochondria where it is a potent inducer of cytochrome c release (5–8). Recombinant mouse Bid was incubated in the presence of lysosomal extract and the products resolved by SDS-PAGE along with Caspase 8-cleaved Bid for comparison (Fig. 5). A 14-kDa cleavage product of Bid resulted from lysosome extract exposure, with the N-terminal sequence SPNGGRIEPD, showing that the lysosomal extract cleaves Bid at Arg$^{65}$ (Fig. 5B).

It was then important to confirm whether the Bid fragment resulting from lysosomal extract exposure had activity on mi-
To test this, mitochondria were isolated from rat cardiac tissue and incubated in the presence of either lysosomal extract alone, Bid alone, Bid and lysosomal extract, or Triton X-100, the last possibility representing total cytochrome c content (Fig. 6A). Uncleaved Bid was able to induce low levels of cytochrome c release at Bid concentration exceeding 100 nM (data not shown) but no detectable release at 1 nM. In contrast, in the presence of lysosomal extract, Bid at 1 nM induced cytochrome c release from mitochondria. Lysosomal extract in the absence of Bid had no effect on cytochrome c release. Thus, lysosome-cleaved recombinant Bid induced cytochrome c release to levels similar to that seen for Caspase 8-cleaved Bid.

**FIG. 5. Effect of lysosomal extract on mouse Bid.** A indicates the processing of recombinant mouse Bid by lysosomal extract as shown by electrophotography followed by Coomassie Blue staining. A positive control of caspase 8-cleaved Bid is shown for comparison. The lysosome-cleaved product indicated by an arrow was subjected to N-terminal sequencing to yield the sequence SFNQGRIEPD. The location of the lysosome cleavage site is shown in B, with the locations of the caspase 8 and granzyme B sites shown for comparison.

**FIG. 6. Cytochrome c release following lysosomal cleavage of recombinant or endogenous Bid.** In A, mitochondria (3 µg of protein at 1 mg/ml concentration) either in the presence or absence of BID (10 nM) and varying volumes of lysosomal extracts were incubated for 40 min at 30 °C. All volumes were supplemented to 25 µl with CMRM, pH 7.5. The pellets and supernatants were collected, and the latter was resolved by SDS-PAGE followed by Western blotting using an anti-cytochrome c antibody. The signals were detected by ECL, and the film was exposed for 30 s. Incubation with Triton X-100 (0.5%; TX) represented total cytochrome c content. The first four lanes represent control trials demonstrating that neither uncleaved Bid nor lysosomal extract alone is capable of inducing cytochrome c release. Incubation of mitochondria with both Bid and increasing amounts of lysosomal extract (right three lanes) induced release of at least 50% of cytochrome c when compared with mitochondria lysed with Triton X-100. B shows similar experiments conducted in cytosolic extracts of mouse hepatocytes, either wild type (Bid^+/+^) or Bid-deficient (Bid^-/-^). In A, 30 µl of cytosol prepared from either wild type (Bid^+/+^) or bid-deficient (Bid^-/-^) mouse hepatocytes were incubated with either 200 ng of recombinant Caspase 8 or 1 to 6 µl of lysosomal extracts in the presence of 5 mM DTT at 37 °C for 30 min. The reaction mixture was then admixed with 15 µg of liver mitochondria at 30 °C for 60 min. The supernatants were then separated from the mitochondria by centrifugation and subjected SDS-PAGE, followed by Western blotting using an anti-cytochrome c antibody.

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Took this, mitochondria were isolated from rat cardiac tissue and incubated in the presence of either lysosomal extract alone, Bid alone, Bid and lysosomal extract, or Triton X-100, the last possibility representing total cytochrome c content (Fig. 6A). Uncleaved Bid was able to induce low levels of cytochrome c release at Bid concentration exceeding 100 nM (data not shown) but no detectable release at 1 nM. In contrast, in the presence of lysosomal extract, Bid at 1 nM induced cytochrome c release from mitochondria. Lysosomal extract in the absence of Bid had no effect on cytochrome c release. Thus, lysosome-cleaved recombinant Bid induced cytochrome c release to levels similar to that seen for Caspase 8-cleaved Bid.

**Reduced Cytochrome c Releasing Activity in bid-deficient Cytosol**—To further confirm that Bid plays an important role as the substrate of lysosomal enzymes in cytochrome c release, we prepared cytosolic extracts from wild type and bid-deficient hepatocytes. When these cytosols were incubated with the lysosomal extracts at different doses, the wild type extracts demonstrated a significantly stronger potency than the bid-deficient extracts to yield a cytochrome c releasing activity (Fig.
6B). The lysosomal extracts were able to activate this activity in a dose-dependent manner, which was comparable with that generated by recombinant caspase 8. In contrast, a modest level of cytochrome c releasing activity was induced in bid-deficient cytosols only when severalfold more lysosomal extracts were used. These results indicated that although Bid might not be the only substrate of lysosomal enzymes that could be activated to induce cytochrome c, it was certainly a major one.

**DISCUSSION**

The concept of lysosomal constituents participating in cellular pathology and degeneration was originally proposed by De Duve (52). Since that time, the critical role of apoptosis in executing cell death under normal and pathologic conditions has become clear. Multiple studies have attempted to address the role of lysosomes in triggering apoptosis. In speaking of lysosomes and cell death, most investigators do not distinguish between primary lysosomes (the temporal store of transient digestive enzymes) and endosomes (the location of protein degradation). Our in vitro system also does not distinguish between the two organelle types, and they are probably a mixture of each. Both organelles contain some specific and many rather nonspecific proteases that participate in general protein digestion and more specific digestion for loading onto major histocompatibility complex class II for antigen presentation.

Evidence for the participation of lysosomes in cell death comes from several sources. Oxidative stress, for example, is generally associated with mitochondrial permeability transition (4), yet it is also readily associated with lysosomal rupture. Thus, cell death following oxidative stress caused by naphtha-zarin (53) and hydrogen peroxide (54) has been linked to lysosomal rupture, as has the death of cultured microglia in response to 6-hydroxydopamine (55), and PC12 cells in response to serum deprivation (56). Natural insults that damage lysosomal membrane integrity include the Alzheimer Aβ1–42 amyloidogenic fragment that accumulates in lysosomal compartments (57) preceding cell death (58). The mechanisms of death induced by lysosomes have previously been linked to direct pro-caspase activation by lysosomal proteases (12), although there is some disagreement because others were not able to see this direct activation (15). Interestingly, Vandenabeele’s group (14, 15) found that lysosomal proteases, and notably cathepsin B, could efficiently process pro-caspases 1 and 11 but could only weakly process the precursors of caspases 2, 3, 6, 7, and 14. Thus, there could be a case for direct activation of inflamma-

tory caspases (1 and 11) but not the apoptotic ones. Therefore, our main goal was to re-examine the hypothesis that lysosomal proteases may lead directly to pro-caspase activation, in search of a mechanistic explanation for lysosome-mediated cell death.

Previous studies have mainly addressed cleavage of pro-
caspases by lysosomal proteases and not their activation. How-
ever, because we have demonstrated that observations of cleav-
age alone can be misleading as a readout of activation, at least for other proteases (59), we focused here on direct measure-
ments of caspase activation. We were surprised to see that the candidate lysosomal proteases and extracts of mouse lysosomes were only able to carry out minimal direct caspase activation, even at very high concentrations. It is possible to see how one may obtain the impression that lysosomal proteases activate caspase zymogens, because slight increases in caspase activity were detected when cathepsin B or X or lysosomal extracts were added to NT2 cytosolic extracts (Figs. 3 and 4, insets). However, when these are compared with the rate achieved by granzyme B, the activities pale into insignificance. Some of the lysosomal proteases, notably cathepsins B and L, would have been rapidly inactivated at the test pH of 7.2, but this was taken into account by control experiments at a more permissive pH of 6.0. Interestingly, there seems to be nothing prohibiting direct activation by members of the papain family, of which all the cathepsins in this study were members, because the homol-
gous protease cruzipain did directly activate pro-caspases 3 and 7, albeit rather weakly. Therefore, we are left with the speculation that the endogenous lysosomal proteases have been negatively selected against for direct caspase activation.

The triggering mechanism of death must have another ex-
planation. It is certainly possible that lysosomal death may sometimes be more necrotic than apoptotic (60), yet there is plenty of evidence that definitive morphology and biochemistry of apoptosis is activated in experimental paradigms of lysosom-
dysfunction (12, 15, 56, 61). Accordingly, we looked else-
where for initiators of apoptosis that would require proteolysis for their activation. The best characterized example is Bid (5–8), and it is clear from our studies with recombinant Bid and endogenous material that this protein can play a role as the lysosomal protease target. Indeed, the dampened release of cytochrome c in extracts from hepatocytes of mice ablated for the bid gene confirms this conjecture. Bid may not be the only cytosolic trigger for lysosome-mediated cytochrome c release, but it is clearly one of them.

The lysosome is a rich source of proteolytic enzymes, and one...
or more of these proteases cleave and activate Bid. The activation was not prevented by pretreating lysosomal extracts with E-64, the broad range inhibitor of the lysosomal proteases cathepsins B, H, K, L, S, and X (data not shown). Therefore, the activity is unlikely to be due to these proteases or a close homolog. The cleavage site (Arg<sup>65</sup>) within Bid was unique, at least as far as could be deduced by Edman degradation, yet cleavage after Arg does not necessarily mean that the protease(s) is Arg-specific. It is equally possible that the protease(s) perceives other determinants in the immediate vicinity. Interestingly, although indirect, evidence places lysosomal aspartic proteases, including cathepsin D in certain apoptosis pathways (53, 62, 63), although cathepsin D itself would not seem to have the desired substrate specificity to recognize the Bid target sequence because it prefers to cleave between hydrophobic residues (64). One clue to the possible identity of the lysosomal protease comes from work on an inherited form of progressive myoclonus epilepsy, where the defect is a complete deficiency in stefin B (65). The disease has been modeled by ablating the mouse stefin B gene, which resulted in ataxia, myoclonic epilepsy and cerebellar apoptosis (66). Thus, stefin B, which inhibits many lysosomal cysteine proteases in vitro (51, 67), is required to avoid pathologic cerebellar apoptosis with the likely explanation that it normally regulates protease(s) that cause the defect. Because we show that stefin B, or other cystatins for that matter, do not inhibit caspases 3, 6, 7, or 8, we concur that stefin B, given its in vitro specificity, may act as an inhibitor of pro-apoptotic lysosomal protease(s) (66).

Although Bid cleavage is the most likely route for lysosome-directed apoptosis, we do not rule out other mechanisms. For example, cleavage of the extended loop segment of Bcl-X<sub>L</sub> by caspases has been demonstrated to accelerate cytochrome <em>c</em> release in vitro (68), and it is possible that lysosomes may also target the same loop. Nevertheless, this would be of secondary importance given the largely impaired cytochrome <em>c</em> release in Bid-deficient cytosols. It is also possible that lysosomal proteases that are not released by the conditions used in our study may directly activate caspases, although such entities are still hypothetical.

The mechanism that Bid uses to induce apoptosis is still unclear, although it is likely that removal of the N-terminal helices 1 and 2 allows the protein to translocate from the cytosol to the mitochondria where it promotes cytochrome <em>c</em> helices 1 and 2 allows the protein to translocate from the mitochondria. Although it is likely that removal of the N-terminal helices 1 and 2 allows the protein to translocate from the cytosol to the mitochondria where it promotes cytochrome <em>c</em> helices 1 and 2, the N-terminal helices 1 and 2 may be required for translocation in vitro. The presence of the N-terminal helices is required for the protein to be targeted to the mitochondria. This relocation of the mitochondrial membranes may be promoted by an increase in hydrophobicity because loss of the N-terminal helices results in a protein that is predicted to have an increased hydrophobic surface area, and this form may favor membrane insertion (31, 69). Alternatively, the cleavage also results in an increased exposure of the BH3 domain, which may promote protein-protein interactions that affect other proteins involved in cytochrome <em>c</em> release, particularly Bak (70) and also Bax (71). Regardless of the mechanism, proteolytic cleavage seems to be essential. Activated caspase 8 cleaves Bid at Asp<sup>59</sup> (6, 7), granzyme B cleaves at Asp<sup>75</sup> (6), and lysosomes cleave at Arg<sup>65</sup> (Fig. 7). These sites encompass a region of the protein that is highly flexible because it does not adopt a fixed conformation as determined by NMR (69, 72). Such regions are excellent targets for proteolysis, because most proteases require flexible protein loops to adapt to their substrate clefs (73). We speculate that the high mobility of this region allows general proteolysis to occur, not just the selective proteolysis of the highly specific caspase 8 and granzyme B. Thus, we propose Bid to be a general sensor of proteolysis by endopeptidases and that the loop shown in Fig. 7 acts as a “bait loop,” allowing cells to respond to adventitious and potentially damaging proteolysis by triggering the built-in apoptotic suicide program. Indeed, this property would not be restricted to lysosomal proteases but may also encompass proteases from other organelles, or even pathogens, that inappropriately enter the cytosol during pathologic episodes.

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Lysosomal Pathways to Apoptosis

Lysosomal Protease Pathways to Apoptosis: CLEAVAGE OF Bid, NOT PRO-CASPASES, IS THE MOST LIKELY ROUTE
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