# Selective Disruption of Lysosomes in HeLa Cells Triggers Apoptosis Mediated by Cleavage of Bid by Multiple Papain-like Lysosomal Cathepsins*

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Increasing evidence suggests that lysosomal proteases are actively involved in apoptosis. Using HeLa cells as the model system, we show that selective lysosome disruption with L-leucyl-L-leucine methyl ester results in apoptosis, characterized by translocation of lysosomal proteases into the cytosol and by the cleavage of a proapoptotic Bcl-2-family member Bid. Apoptosis and Bid cleavage, but not translocation of lysosomal proteases to the cytosol, could be prevented by 15 $\mu$M L-*trans*-epoxysuccinyl(OEt)-Leu-3-methylbutyramide, an inhibitor of papain-like cysteine proteases. Incubation of cells with 15 $\mu$M N-benzoyloxycarbonyl-VAD-fluoromethyl ketone prevented apoptosis but not Bid cleavage, suggesting that cathepsin-mediated apoptosis in this system is caspase-dependent. *In vitro* experiments performed at neutral pH showed that papain-like cathepsins B, H, L, S, and K cleave Bid predominantly at Arg[65] or Arg[71]. No Bid cleavage was observed with cathepsins C and X or the aspartic protease cathepsin D. Incubation of full-length Bid treated with cathepsins B, H, L, and S resulted in rapid cytochrome *c* release from isolated mitochondria. Thus, Bid may be an important mediator of apoptosis induced by lysosomal disruption.

Apoptosis is the major mechanism by which multicellular organisms remove superfluous, infected, damaged, or potentially dangerous cells (1). Caspases, a family of cysteine proteases that reside in an inactive zymogen form in the cytosol of virtually every cell, play a major role in apoptotic execution. Caspase activation, a critical event in apoptosis progression, can be achieved in several ways, including the extrinsic pathway, characterized by death receptor-mediated recruitment and activation of apical caspase-8, and the intrinsic pathway, characterized by assembly of cytosolic (APAF-1) and mitochondrial (cytochrome *c*) factors with subsequent activation of apical caspase-9 (2). These two pathways converge at the level of activation of executioner caspases-3 and -7 (1). The extrinsic

and the intrinsic pathways are connected through Bid, a proapoptotic BH3-only Bcl-2 family member. In certain cell types, caspase-8 has been shown to cleave Bid after Asp[59], thereby removing the two N-terminal helices and converting Bid from a latent to a strongly proapoptotic molecule, capable of inducing cytochrome *c* release through the action of Bax or Bak and subsequent activation of caspase-9 (3). Bid is also cleaved during apoptosis by granzyme B, a serine protease from cytotoxic T lymphocytes (4, 5); by calpains (6–8); and by lysosomal proteases (9). Although all of the cleavage sites differ from the caspase-8 cleavage site, they map to the same region of Bid, corresponding to a loop between $\alpha$-helices 2 and 3.

In addition to caspases, increasing evidence suggests a role for lysosomal proteases in apoptosis, including clearance of infected cells and various pathologies, such as cancer and neurodegeneration (reviewed in Refs. 10 and 11). Lysosomes contain a number of proteases, among them the aspartic protease cathepsin D and the cysteine protease cathepsin B, which are the most abundant and have most often been reported to be involved in apoptosis induction (12–17). The other abundant lysosomal proteases are the family of papain-like cathepsins related to cathepsin B (cathepsins H, L, K, S, C, X, V, W, F, and O; Refs. 18 and 19). During apoptosis induced by sphingosine and lysosomotropic reagents, cathepsins were found to be translocated to cytosol prior to mitochondrial damage and cytochrome *c* release, thereby inducing apoptosis by mechanisms that have not yet been clarified (reviewed in Refs. 10 and 11).

In some of the models, cathepsins have been suggested to act independently of caspases, whereas in other models apoptosis was suggested to be caspase-mediated (reviewed in Refs. 10 and 11). Direct caspase activation by the cathepsins is unlikely because a number of the cathepsins failed to activate proapoptotic caspases-3 and -7 (9, 20). Therefore, additional cytosolic factor(s) were suggested to be involved (15). One of the most attractive cytosolic targets of the cathepsins is Bid, which has already been suggested to be an important mediator of lysosomal protease-mediated apoptosis (9, 21). However, the lysosomal protease(s) performing the cleavage have yet to be identified.

Our studies were therefore aimed toward understanding the molecular mechanisms by which lysosomal proteases induce apoptosis with the major focus on Bid. Using the lysosomotropic agent LeuLeuOMe,[1] we show that disruption of lysosomes re-

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[1] The abbreviations used are: LeuLeuOMe, L-leucyl-L-leucine methyl ester; AC27P, acetyl-calpastatin 27-peptide; E-64, L-*trans*-epoxysuccinyl-Leu-amido-(4-guanidino) butane; E-64d, L-*trans*-epoxysuccinyl-(OEt)-Leu-3-methylbutylamide; Z, N-benzoyloxycarbonyl; fmk, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulphonate.
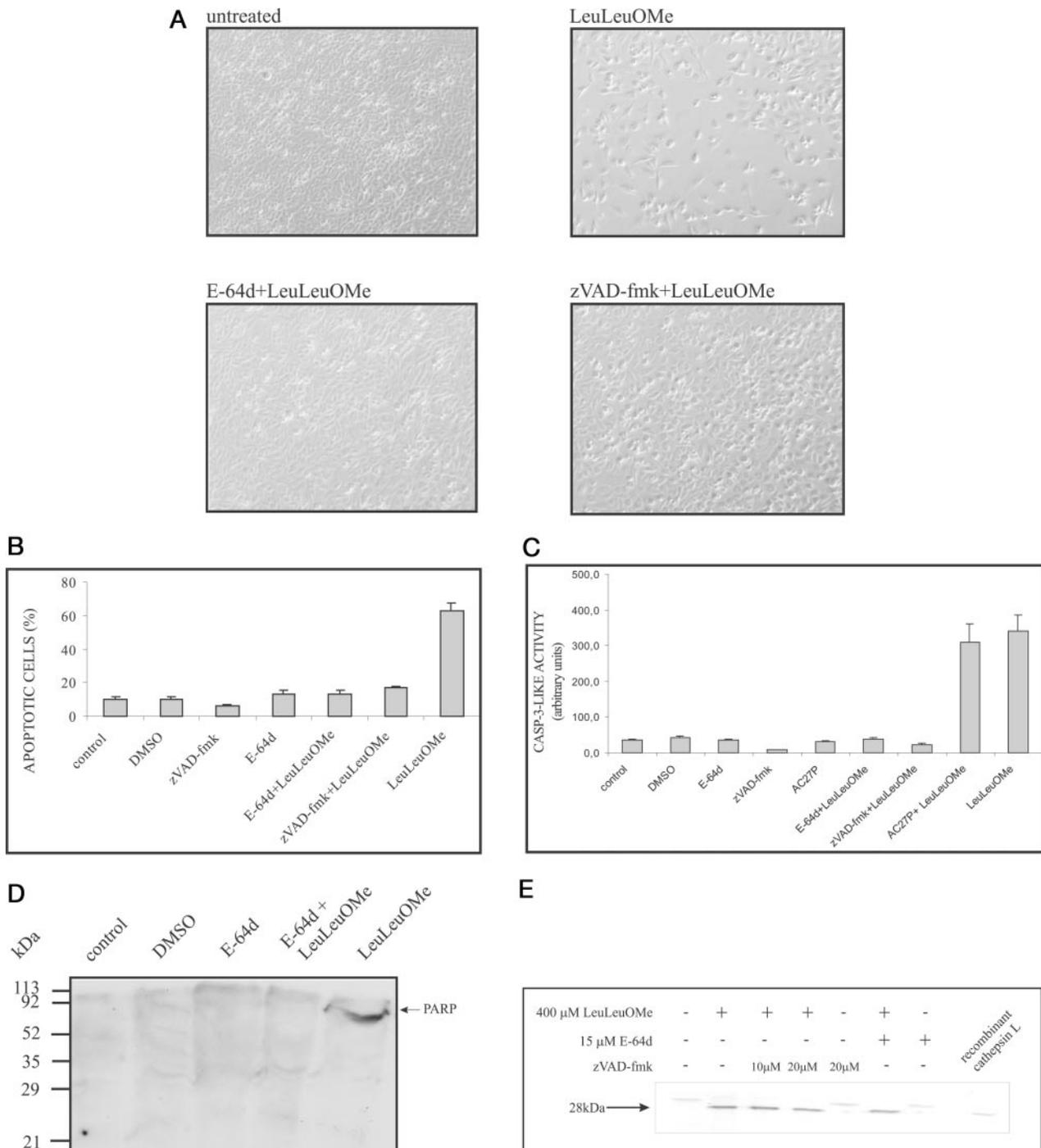
Fig. 1. **Apoptosis induction by LeuLeuOMe in HeLa cells.** All details are given under "Experimental Procedures." *A*, following treatment with 500 $\mu$M LeuLeuOMe cells shrunk, rounded, and detached from the surface as seen by light microscopy. The majority of these morphological changes were prevented by E-64d and Z-VAD-fmk. *B*, phosphatidylserine exposure was largely prevented by pretreatment of cells with E-64d or Z-VAD-fmk when compared with the control. The *numbers* indicate the percentages of cells undergoing apoptosis. *C*, a large increase in DEVD-ase activity was observed after treatment of cells with LeuLeuOMe, which was abolished by pretreatment of cells with E-64d or Z-VAD-fmk, but not with AC27P. *D*, PARP cleavage was observed only in cells treated with LeuLeuOMe. *E*, LeuLeuOMe induced release of cathepsin L from lysosomes in HeLa cells. *DMSO*, dimethyl sulfoxide.

sults in translocation of lysosomal proteases to the cytosol and induction of apoptosis through a caspase-dependent mechanism, involving Bid cleavage by papain-like cysteine proteases. To identify the cleaving protease(s), a detailed investigation has been performed using cathepsins B, H, L, S, K, X, C, and D.
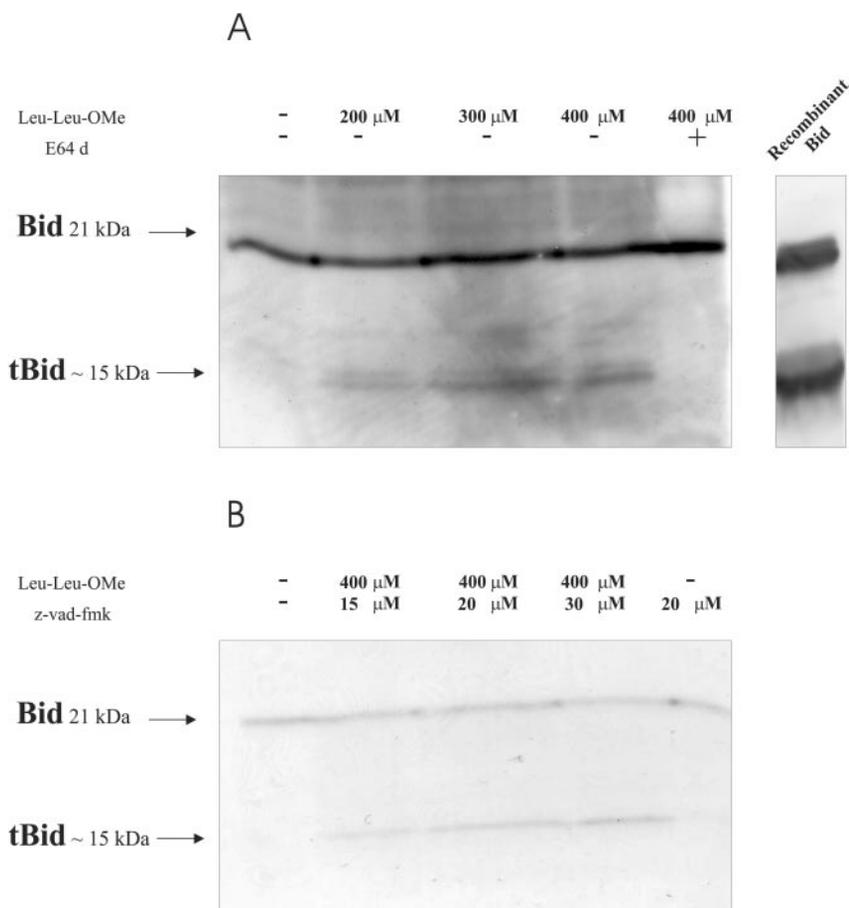
<div style="text-align:center">EXPERIMENTAL PROCEDURES</div>

*Proteins*—Recombinant human cathepsins B, L, and K, human caspase-8, and mouse Bid were prepared according to published procedures (22–26), whereas cathepsins H, X, and C were purified from porcine liver (27), human liver (28), and human kidney (29), respectively. All of the cathepsins were active site-titrated as described elsewhere (30).

*Materials*—Human embryonic kidney cell line 293 and human adenocarcinoma cell line HeLa were purchased from ATCC. Polyclonal anti-Bid and anti-cathepsin L antibodies were prepared using standard protocols. Monoclonal antibodies specifically recognizing the cleaved form of PARP were from Promega, and cytochrome *c* antibodies were from PharMingen. Horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG antibodies, fetal calf serum, Percoll, *p*-iodonitrotetrazolium violet, 4-methylumbelliferyl-2-acetamido-2-dexy-D-

## A



## B

FIG. 2. **Bid cleavage in LeuLeuOMe-treated HeLa cells.** Caspase-8 cleaved recombinant Bid was used as a positive control. Bid cleavage was observed in cells treated with LeuLeuOMe, which could be completely abolished by pretreatment of cells with 15 $\mu$M E-64d (*A*) but not with Z-VAD-fmk (*B*).

glucopyranoside, cytochrome *c*, E-64, E-64d, and pepstatin A were obtained from Sigma. The caspase substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluorometylcoumarin, the calpain-specific inhibitor AC27P, and the lysosomotropic reagent LeuLeuOMe were purchased from Bachem. All of the nucleotide primers were purchased from MWG Biotech AG. All other chemicals were of analytical grade.

*Cell Culture and Apoptosis Triggering*—HEK 293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% (v/v) fetal calf serum, streptomycin, and penicillin in 5% $CO_2$ atmosphere at 37 °C.

HeLa cells from confluent 10-cm dishes were split in a 1:6 ratio to yield $6 \times 10^6$/well of 6-well plates. After 10 h E-64d or Z-VAD-fmk was added to a final concentration of 15 $\mu$M, followed by overnight incubation. The next day LeuLeuOMe was added in a negligible volume to a final concentration of 400–500 $\mu$M. After 24 h the cells were viewed by light microscopy (Olympus M021, magnification 40) and harvested by trypsinization. In control experiments, $Me_2SO$ was added in a volume corresponding to the volume of the E-64d. Because E-64 and its derivatives also inhibit calpain (30), an additional experiment was performed using 50 $\mu$M specific calpain inhibitor AC27P (8).

*Cell Extracts*—Initially, the cells were washed twice in ice-cold phosphate-buffered saline. After this step, the procedures for preparing cytosolic or total cell extracts differed. To prepare cytosolic extracts, the cells were washed twice in the isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES) containing a protease inhibitor mixture including pepstatin A, aprotinin, leupeptin, E-64, and bestatin (Sigma). The cells were homogenized by 20 passages through a 25 G ($0.5 \times 16$) needle. Nuclei and unbroken cells were removed by centrifugation at $500 \times g$ for 5 min, followed by two consecutive centrifugation steps, the first at $10,000 \times g$ for 5 min and the second at $100,000 \times g$ for 1 h. Use of the inhibitors ensured that any unwanted proteolysis, especially the proteolysis by papain-like cathepsins, was prevented during the preparation of the extracts.

Total cell extracts were prepared from cells by lysing them in 50 $\mu$l of RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 1 mM EDTA). Following 10 min of incubation on ice, insoluble material was removed by centrifugation at 14,000 rpm for 10 min. In both cases, the supernatants were transferred to fresh test tubes and either used immediately or stored at $-80$ °C. Total protein concentration was determined using the Bradford assay (Bio-Rad).

*Immunodetections*—After treatment, aliquots of cell lysates (80 $\mu$g of protein based on the Bradford assay) were resolved by 15% SDS-PAGE. Bid, tBid, cathepsin L, the cleaved form of PARP, and cytochrome *c* were then determined by Western blotting and development with ECL according to the manufacturer's instructions (Amersham Biosciences).

*Flow Cytometry*—To measure phosphatidylserine exposure, $10^6$ HeLa cells were labeled with annexinV-PE and 7-amino-actinomycin D (BD Biosciences) according to the manufacturer's instructions. The cells were then subjected to fluorescence-activated cell sorter analysis using a FACScalibur flow cytometer (BD Biosciences) and CellQuest software.

*Measurement of DEVD-ase Activity*—Cell lysates (100 $\mu$g of protein) were transferred into the 96-well plate and filled to the final volume of 90 $\mu$l with the caspase buffer (100 mM HEPES, 200 mM NaCl, 0.2% (w/v) CHAPS, 20% (w/v) sucrose, 2 mM EDTA, 20 mM dithiothreitol, pH 7.0). Following 15 min of incubation at 37 °C, acetyl-DEVD-7-amino-4-trifluorometylcoumarin was added to a final concentration of 100 $\mu$M, and DEVD-ase activity was measured continuously in an LS50B fluorimeter with plate reader attachment (PerkinElmer Life Sciences) at excitation and emission wavelengths of 400 and 505 nm, respectively.

*Isolation of Mouse Liver Lysosomes*—Lysosomes were isolated from mouse liver essentially as described previously (9). The fractions collected were assayed for mitochondrial and lysosomal contents using *p*-iodonitrotetrazolium reductase and $\beta$-hexosaminidase as markers (31, 32), respectively. The fractions containing pure lysosomes were pooled and washed twice in an equal volume of lysosomal buffer (250 mM sucrose, 20 mM HEPES, pH 7.2). The lysosomes were then aliquoted and stored at $-80$ °C for further use.

Lysosomal extracts were prepared from lysosomes by three cycles of freezing and thawing. Pellets containing lysosomal membranes were removed by 5 min of centrifugation at 14,000 rpm. The supernatant was kept on ice and used freshly.

*In Vitro Cleavage of Bid and Analysis of Cleavage Products*—To prevent unwanted inactivation, the enzymes were kept in their normal storage buffers (100 mM phosphate buffer, pH 6.0, for lysosomal extract and cathepsins B and C; 100 mM phosphate buffer, pH 6.8, for cathepsin
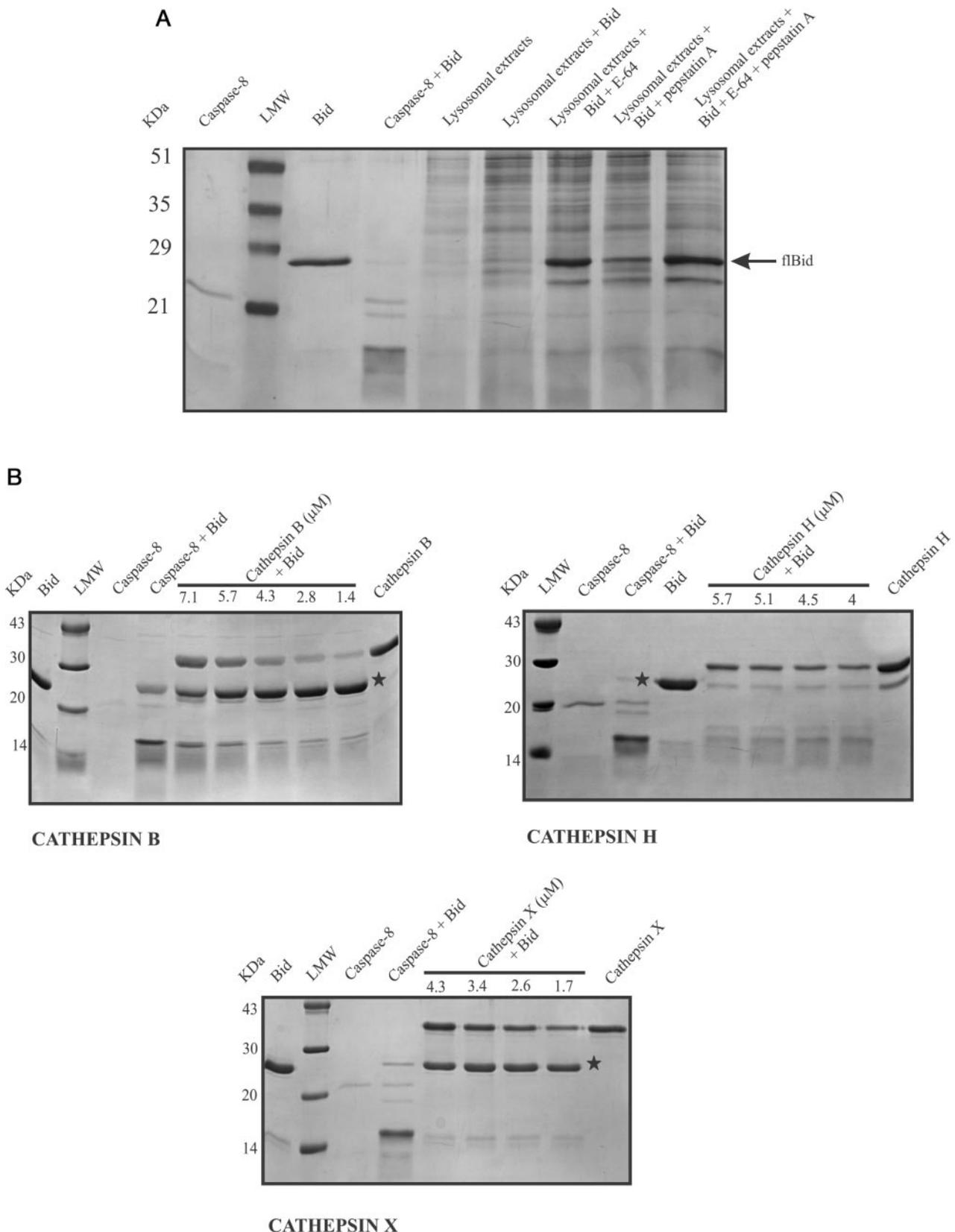
FIG. 3. *In vitro* **cleavage of recombinant Bid at pH 7.2 and 37 °C.** All of the experimental details are given under "Experimental Procedures." *A*, lysosomal extracts were incubated with Bid in the presence or absence of E-64 (20 $\mu$M), pepstatin A (500 $\mu$M), or combination of both. Bid was completely degraded in the absence of inhibitors, whereas degradation could be almost completely prevented in the presence of E-64. Caspase-8-cleaved Bid was used as a positive control. Recombinant Bid, caspase-8, and lysosomal extract (half the amount of the loading in *lanes 6–9*) were used as negative controls. *B*, cleavage of Bid by cathepsin B, cathepsin X, and cathepsin H. A concentration-dependent cleavage of Bid was observed following incubation with cathepsin B, resulting in an additional band migrating at ~15 kDa. No cleavage of Bid was observed in the presence of cathepsin X. Almost complete degradation of full-length Bid was observed following incubation with cathepsin H. All three cathepsins, caspase-8, and recombinant Bid were used as negative controls, whereas caspase-8-cleaved Bid was used as a positive control The additional band present in the *lanes* with Bid results from spontaneous Bid degradation. Full-length Bid is marked with an *asterisk. LMW*, low molecular weight standards.

TABLE I
*Cleavage sites of lysosomal extracts and individual cathepsins in Bid*

| Protease | Cleavage sites | |
|---|---|---|
| | Major | Minor |
| Lysosomal protease extract | Arg[65] | |
| Cathepsin B | Arg[65] | Ser[66], Phe[67] |
| Cathepsin K | Arg[65] | Gly[12] |
| Cathepsin L | Arg[65] | |
| Cathepsin S | Arg[65] | Gly[12], Tyr[47], Gln[57] |
| Cathepsin H | Arg[71] | Ser[6], Gly[12], Tyr[47] |
| Cathepsin X | | |
| Cathepsin C | | |
| Cathepsin D | | |

H; 100 mM phosphate buffer, pH 7.0, for cathepsin S; 50 mM acetate buffer, pH 5.1, for cathepsin X; 340 mM acetate buffer, pH 5.5, for cathepsins L and K; and 340 mM acetate buffer, pH 4.2, for cathepsin D) containing 1 mM EDTA. Cathepsins and lysosomal extracts were then added in a small volume ($\leq 5$ μl) to the same buffers with pH raised to pH 7.2, containing 2 mM dithiothreitol (final concentration) and 20 μM Bid to a final volume of 35 μl. As verified in separate experiments, this ensured that the final pH (7.2) remained unchanged. Caspase-8 incubated in caspase buffer (see above) was used as a positive control. Following 1 h of incubation at 37 °C, the reactions were terminated by the addition of 5 μl of 1 M dithiothreitol and SDS-PAGE loading buffer and boiling for 5 min. In another experiment, the lysosomal extracts were pretreated with E-64 (final concentration, 20 -300 μM) and/or pepstatin A (500 μM) for 30 min prior to addition of Bid. The reaction products were analyzed by SDS-PAGE on 15% gels, followed by Coomassie Blue staining or, alternatively, transferred to the polyvinylidene difluoride membranes, stained for 1 min with Coomassie Blue (Bio-Rad), destained, and washed overnight with distilled water. The appropriate bands were then excised, and the N-terminal sequences of the Bid cleavage products were determined by Applied Biosystems 492 amino acid sequencer.

*Isolation of Mouse Heart Mitochondria*—Mitochondria were isolated from murine hearts using the modified procedure of Ott *et al.* (33). The mitochondria were resuspended in an equal volume of MSH buffer (250 mM sucrose, 10 mM HEPES, 2 mM $KH_2PO_4$, 5 mM sodium succinate, 10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM $MgCl_2$), pH 7.5, kept on ice, and used within 30 min to minimize the risk of disruption.

*In Vitro Assay for Cytochrome c Release from Purified Mitochondria*—Recombinant Bid (7 μg) was incubated with cathepsins B, L, H, S, and K (final concentrations, 50 nM to 3 μM) in 5 mM HEPES buffer, pH 7.2, containing 250 mM mannitol, 0.5 mM EGTA, 0.1% (w/v) bovine serum albumin and 5 mM dithiothreitol, in a final volume of 30 μl for 40 min at 37 °C prior to the addition of fresh murine mitochondria (20 μg of protein). Following an additional 5-min incubation at 37 °C, the supernatants were separated from mitochondria by centrifugation at 9000 rpm for 5 min at 4 °C. Cytochrome *c* was then detected by Western blotting. In a control experiment, a pellet containing mitochondria was treated with 2% (v/v) Triton X-100.

*Preparation of Bid Variants in pcDNA3*—Full-length Bid (flBid) was excised from pGEX4Ti vector using EcoRI and XhoI sites and cloned into the pcDNA3 vector at the same sites. Truncated Bid variants were generated by PCR using primers that bound to flBid DNA. The following primers were used: 5'-AGCGAATTCATGTGGGAGGCAGA-3' for tBid-Tyr[47], 5'-TTGGAATTCATGACAGACGGCAGC-3' for tBid-Gln[57], 5'-ATAGAATTCATGGGCAGCCAGGC-3' for tBid-Asp[59], 5'-TCCGAATTCATGTCCTTCAACC-3' for tBid-Arg[65], and 5'-TCGGAATTCATGATAGAGCCAGA-3' for tBid-Arg[71]. The reverse primer was always 5'-CCGCTCGAGTCAGTCCATC-3'. All of the sequences were verified by DNA sequencing using an Abi Prism 310 automated DNA sequencer (PerkinElmer Life Sciences). The fragments were then inserted into pcDNA3 using EcoRI and XhoI sites. To check whether the new constructs generate functional protein, tBid and flBid proteins were expressed *in vitro* using the Promega TnT-coupled transcription-translation system and radiolabeling with [$^{35}$S]methionine (Amersham Biosciences). The efficiency of expression was monitored by analyzing the translate by SDS-PAGE and autoradiography.

*Transfections of Cells with tBid Variants*—Transfections of HEK 293 cells were performed in 12-well plates. The cells were transfected with equal amounts of pcDNA3/tBid constructs using LipofectAMINE™ 2000 (Invitrogen) according to the instructions of the manufacturer. pcDNA3 and pcDNA3/flBid were used as negative controls, whereas the pcDNA3/caspase-8 construct was used as a positive control. Transfection efficacy was controlled by cotransfections with EGFP.

RESULTS

*Characterization of LeuLeuOMe-triggered Cell Death in HeLa Cells*—LeuLeuOMe has been shown to induce apoptosis in a variety of cells, including natural killer cells and macrophages, by selective disruption of lysosomes. The compound enters lysosomes by endocytosis. LeuLeuOMe accumulates in lysosomes followed by conversion to its membranolytic form (LeuLeu)$_n$-OMe ($n \geq 3$) by the transesterase activity of another lysosomal protease, cathepsin C (dipeptidyl peptidase I) (34). Finally, lysosomal membrane integrity is lost, resulting in caspase activation and apoptosis (35). Various cells exhibit different sensitivity toward LeuLeuOMe, the most sensitive being the cytotoxic lymphocytes (36, 37).

Because of their reasonably high content of Bid and various cathepsins including cathepsin C, we chose HeLa cells as a cellular model to investigate this process. Initially, LeuLeuOMe in the concentration range 50–1000 μM was tested for its ability to induce cell death. The majority of cells died in an apoptotic manner at LeuLeuOMe concentrations of $\geq 300$ μM with an optimum at 400–500 μM. This is in agreement with previous results on human myeloma tumor cell lines U937, HL60, and THP-1 (200–500 μM; Refs. 35 and 36). However, at LeuLeuOMe concentrations $\geq 750$ μM, the cells died with necrotic morphology without any increase in caspase activity (not shown). Therefore, all further experiments were performed at LeuLeuOMe concentrations of 400–500 μM.

Treatment of HeLa cells with 500 μM LeuLeuOMe for 24 h resulted in detachment of cells from the culture dishes and major morphological changes, such as considerable cell shrinkage and rounding (Fig. 1*A*). All of the morphological changes were substantially prevented by pretreatment of cells with a general inhibitor of cysteine cathepsins E-64d at 15 μM or with the pancaspase inhibitor Z-VAD-fmk, also at 15 μM (Fig. 1*A*), suggesting involvement of both cysteine cathepsins and caspases in the process. In the next step, the cells were tested for the surface exposure of phosphatidylserine, another marker of apoptosis, by flow cytometry. Following LeuLeuOMe treatment 62.5% of cells were apoptotic, which could be prevented by pretreating cells with 15 μM E-64d (11.8% apoptotic cells) or with 15 μM Z-VAD-fmk (16.9% apoptotic cells). In a control experiment 10.8% of cells were apoptotic, similar to the result in the presence of E-64d (11.5%) or Z-VAD-fmk (7.5%) alone, which were used as negative controls (Fig. 1*B*). Similarly, DEVD-ase activity (a reporter of general caspase activation) was highly elevated in LeuLeuOMe-treated cells (Fig. 1*C*), in agreement with a major role of caspases in this type of cell death (35). Caspase-3 activation was confirmed by immunodetection of cleaved PARP using the antibodies that specifically recognize the caspase-3-generated truncated form of PARP in LeuLeuOMe-treated cells only (Fig. 1*D*). However, caspase activity could be ablated by pretreating cells either with 15 μM E-64d or 15 μM Z-VAD-fmk (Fig. 1, *C* and *D*) but not by AC27P (Fig. 1*C*), thereby suggesting that calpains are not implicated in this apoptosis model. Finally, translocation of lysosomal proteases to the cytosol was confirmed by Western blot analysis, demonstrating the presence of active cathepsin L in the cytosolic extract of HeLa cells treated with LeuLeuOMe (Fig. 1*E*). Significantly, none of the inhibitors used (E-64d or Z-VAD-fmk) prevented translocation of cathepsin L to the cytosol, strongly suggesting that active cathepsins are required in the cytosol to activate caspases (Fig. 1*E*). Interestingly, in all of the control cells but not in LeuLeuOMe-treated cells, a small band with a slightly higher molecular mass ($\sim$33 kDa), probably corresponding to the inactive zymogen form of cathepsin L, was observed (Fig. 1*E*).

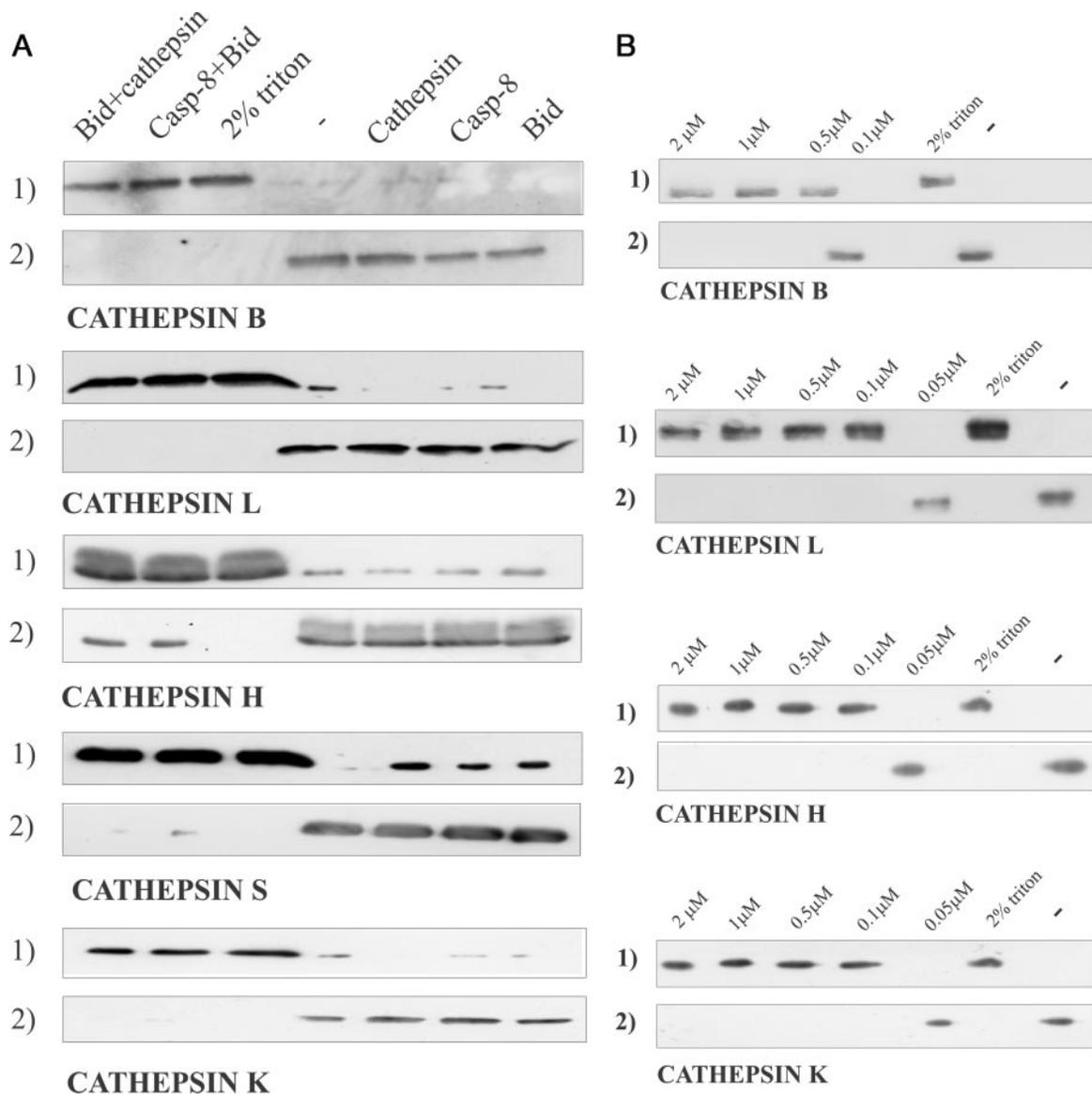*LeuLeuOMe-triggered Apoptosis in HeLa Cells Is Accompanied by Bid Cleavage*—The finding that cathepsins act up-

FIG. 4. **Cytochrome *c* release from mitochondria following Bid incubation with lysosomal proteases and mitochondria at pH 7.2 and 37 °C.** *A*, mitochondria were incubated with cathepsins B, L, H, S, and K in the presence or absence of Bid. Caspase-8 and Triton X-100 were used as positive controls. *Panels 1*, cytosol, *panels 2*, pellet. *B*, dependence of cytochrome *c* release from mitochondria on cathepsin concentration. *Panels 1*, cytosol, *panels 2*, pellet. All other details are given under "Experimental Procedures."

stream of caspases in HeLa cells, where apoptosis was triggered by selective lysosome disruption, raised the question about the molecular mechanism of caspase activation in this model. We focused our studies on Bid, which has been suggested to be a sensor of lysosomal proteases (9, 21). First, the presence of Bid in untreated HeLa cells was confirmed, and Bid was found as a full-length form migrating at ~22 kDa (Fig. 2). Next, HeLa cells were treated for 24 h with LeuLeuOMe (200–400 $\mu$M), which resulted in partial cleavage of Bid and appearance of another band migrating at ~15 kDa. Generation of this truncated (tBid) variant was completely abolished in the presence of E-64d but not Z-VAD-fmk, suggesting that papain-like cathepsins were directly or indirectly responsible for the cleavage.

*In Vitro Cleavage of Recombinant Bid*—Although inhibition of cathepsins blocked Bid cleavage induced by lysosomotropic agents in HeLa cells, this does not necessary indicate that cathepsins directly cleave Bid. To address this question, *in vitro* experiments were performed using recombinant mouse Bid. First, recombinant Bid was incubated at neutral pH with lysosomal extracts, containing 2.3 $\mu$M cathepsins as judged on

the basis of E-64 titration, resulting in complete Bid degradation (Fig. 3*A*). Bid degradation was prevented by preincubation with E-64 ($\geq$15 $\mu$M), suggesting that papain-like cathepsins are responsible for the cleavage, in agreement with results in our cellular model. In contrast, preincubation of the extracts with pepstatin (500 $\mu$M) only partially protected Bid against degradation, suggesting a minor role, if any, for the cathepsin D and related aspartic proteases. Second, to identify Bid cleavage sites by the lysosomal proteases, the experiment was repeated with a lower concentration of lysosomal extracts (not shown). A single cleavage product was found and analyzed by electroblotting and N-terminal sequencing, which revealed that the cleavage occurred after Arg[65], consistent with previous results (9).

Third, Bid was incubated with increasing concentrations of individual cathepsins B, K, L, H, S, C, X, and D (1–8 $\mu$M, depending on the protease). Bid cleavage, which was concentration-dependent, was observed with cathepsins B (Fig. 3*B*), K, L, and S (not shown). Substantial Bid cleavage was also seen with cathepsin H (Fig. 3*B*), whereas no cleavage was observed with cathepsins C, X, and D. N-terminal sequence analyses of
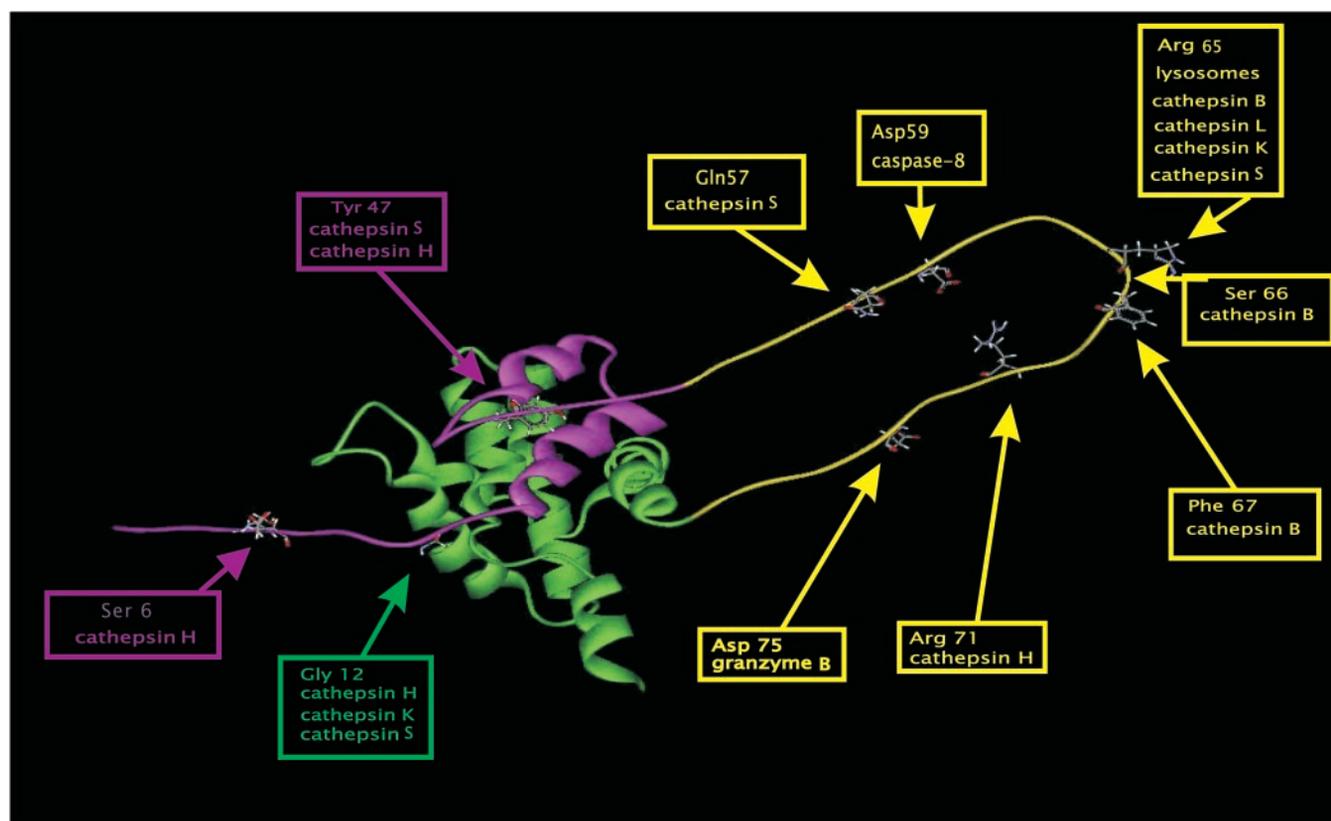
FIG. 5. **Location of protease cleavage sites in Bid.** Bid is shown with a ribbon plot. The N-terminal helices, which are removed to convert to the pro-apoptotic form, are colored *magenta*; the "bait loop," where most of the protease cleavage sites reside, is *yellow*; and the C-terminal part of Bid, which is implicated in progression of apoptosis, is *green*. All of the cleavage sites are marked together with the cleaving proteases. The coordinates were taken from the Protein Data Base (2BID). The figure was prepared using WebLabViewer Lite Version 4.0 (Molecular Simulations Inc.).

the cleavage products identified as the major cleavage sites Arg[65] (cathepsins B, K, L, and S) and Arg[71] (cathepsin H). In addition, several minor cleavage sites were identified (Table I). Caspase-8, which was used as a positive control in all of the experiments, was much more efficient in cleaving Bid than any of the lysosomal proteases (Fig. 3).

*Bid-mediated Cytochrome c Release from Isolated Mitochondria*—Because truncation of Bid acutely enhances its proapoptotic potential (38, 39), we next investigated whether cathepsins B, L, H, S, and K, previously shown to cleave Bid, are capable of facilitating the release of cytochrome *c* from isolated mitochondria. Following a short incubation of mitochondria with Bid and cathepsins, release of all of the cytochrome *c* from mitochondria was observed for all five cathepsins (B, L, S, K, and H; Fig. 4*A*). Similar results were obtained with both positive controls, caspase-8 and the detergent Triton X-100, whereas no cytochrome *c* was released from mitochondria when either Bid or the protease (cathepsins/caspase-8) was omitted from the reactions. Because the above experiments were performed with high cathepsin concentration (3 $\mu$M), which may be nonphysiological, we analyzed the concentration dependence of cathepsins B, L, K, and H (2 $\mu$M to 50 nM), revealing efficient tBID-mediated cytochrome *c* release at cathepsin concentrations that were $\geq$100 nM (Fig. 4*B*).

*tBid Mutants Induce Apoptosis in Transfected Cells*—With the exception of Ser[6] and Gly[12], all other cathepsin cleavage sites on Bid are found within the flexible loop connecting helices 2 and 3 (40, 41), together with the cleavage sites for caspase-8 (Asp[59]) and granzyme B (Asp75) (Fig. 5). To check whether all of the truncated Bid variants (Tyr[47], Gln[57], Arg[65], and Arg[71]) are capable of inducing apoptosis in cells, we prepared pcDNA3 mammalian expression plasmids encoding

these variants of tBid. HEK 293 cells were then transfected with all four cathepsin tBid variants and an Asp[59] tBid variant, which simulates caspase-8 cleavage, as a positive control. A substantial increase in DEVD-ase activity was observed in cells transfected with each of the tBid variants, but not in the cells transfected with full-length Bid or empty pcDNA3 vector, employed as negative controls (Fig. 6*A*). Finally, apoptosis was confirmed by immunodetection of cleaved PARP in cells transfected with each of the tBid variants (Fig. 6*B*). Efficiency of transfection was verified by immunodetection of tBid variants in the cell extracts, confirming comparable levels of protein production (not shown).

DISCUSSION

Until recently, papain-like lysosomal proteases, the cathepsins, were believed to be primarily involved in nonselective intracellular protein degradation in lysosomes, and their function outside lysosomes was ignored because of their instability at neutral pH (reviewed in Refs. 18 and 42). However, cathepsins survive at neutral pH from several minutes (cathepsin L; Ref. 43) to several hours (cathepsin S; Ref. 44), allowing transient activity in the cytosol. Because their intralysosomal concentration can be as high as 1 mM (45), they represent an enormous destructive potential if released from lysosomes. Outside lysosomes, the major regulators of cathepsins activity are their endogenous inhibitors, the stefins (or cystatins), which inhibit accidentally escaped or activated proteases, thereby serving the role of threshold inhibitors similar to IAP (inhibitor of apoptosis), which operate as endogenous inhibitors of caspases (42). Massive lysosomal damage leads to necrosis (reviewed in Ref. 46), suggesting that the inhibitory potential of the cystatins is exceeded. On the other hand, moderate lysoso-
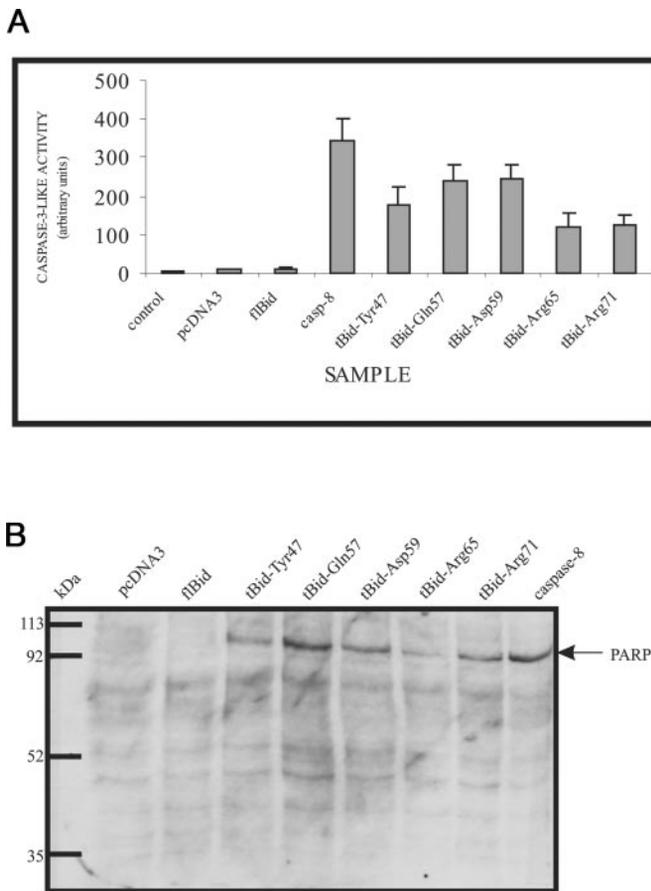
FIG. 6. **Apoptosis in HEK 293 cells transfected with different truncated forms of Bid.** *A*, DEVD-ase activity measured in total cell lysates of transfected cells. *B*, proteolysis of PARP revealed by a cleavage site-specific antibody.



FIG. 7. **Intrinsic apoptotic pathway and involvement of lysosomal proteases.** Following lysosomal damage, caused for example by oxidative stress, cathepsins can translocate to the cytosol, where they cleave Bid, thereby converting it into its proapoptotic form (tBid). tBid induces release of cytochrome *c* and other proapoptotic molecules (Smac/DIABLO, Omi/HtrA2, and AIF) from mitochondria and subsequent caspase-9 activation. Active caspase-9 in turn activates the executioner caspases leading to death substrate cleavage and apoptosis execution. Cathepsins and caspases are additionally regulated by their endogenous inhibitors stefins (cathepsins) and IAPs (inhibitor of apoptosis proteins) (caspases).

mal damage, which may only slightly overcome the inhibitory potential of the cystatins, leads to apoptosis, as shown in several examples (47–49). In stefin B-deficient mice, neuronal apoptosis is observed even in the absence of lysosomal damage (50, 51) as a result of basal flux of lysosomal proteases across lysosomal membranes or incomplete cathepsin trafficking to lysosomes (10).

In this study, we found that apoptosis triggered by LeuLeuOMe was not only caspase-dependent but also cathepsin-dependent, as judged on the basis of inhibitor studies (Fig. 1). Because E-64 and E-64d are not inhibitors of caspases (30), the cathepsins appear to act upstream of caspases in this apoptosis model. Moreover, E-64 is only a very poor inhibitor of cathepsin C (52), which would explain translocation of cathepsins to the cytosol following LeuLeuOMe treatment in the presence of the inhibitor (Fig. 1) and suggests that cathepsin C has no further role in the propagation of apoptosis. Incubation of HeLa cells with the pan-caspase inhibitor Z-VAD-fmk also prevented morphological signs of apoptosis and phosphatidylserine exposure, indicating that caspase activation is required in this model for apoptosis progression. Furthermore, Z-VAD-fmk was used at concentrations that do not significantly inhibit the cathepsins (15 $\mu$M; Ref. 30), suggesting that cathepsins are required only for caspase activation and not for apoptosis execution in this model, in contrast to some previous data on tumor apoptosis induced by tumor necrosis factor (17). Although E-64 and E-64d can also inhibit calpain, the possible involvement of calpain in this apoptosis model has been ruled out by the use of specific calpain inhibitor AC27P (8), which had no effect on caspase activation.

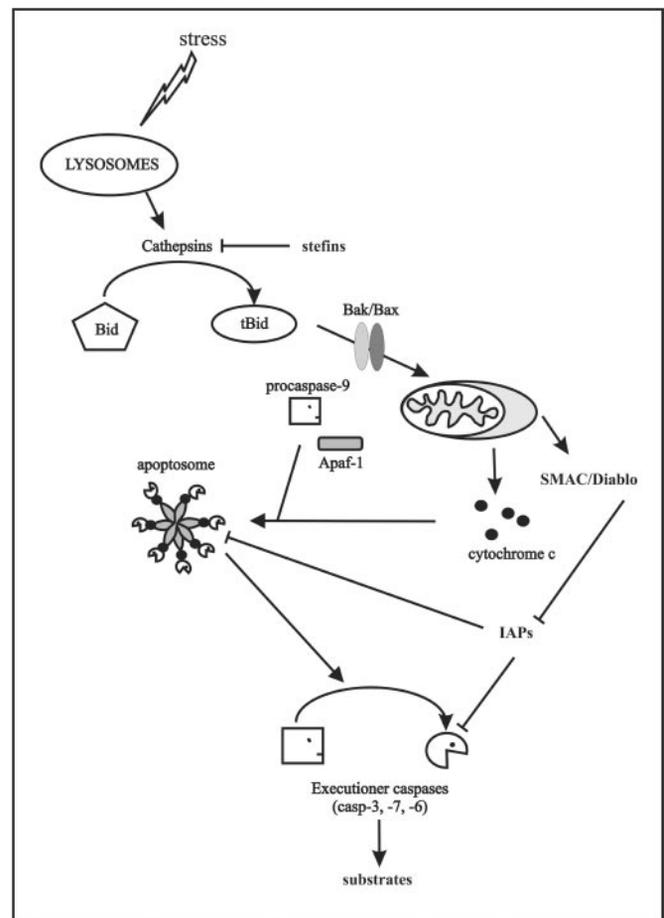Because proapoptotic caspases are not likely to be directly activated by lysosomal proteases (9, 20, 21), a critical point in understanding the molecular mechanism of lysosome-triggered apoptosis was identification of the cellular substrate(s) of cathepsins that could facilitate caspase activation. Likely candidates were proteins from the Bcl-2 family, which are known as critical regulators of the intrinsic pathway of apoptosis (3, 53). Among them Bid, a proapoptotic member of the Bcl-2 family, was chosen as a candidate for several reasons: (i) Bid requires limited proteolysis to become highly proapoptotic (38, 39); (ii) Bid has already been found to be a substrate of lysosomal proteases in lysosomal photodamage model of apoptosis in hepatoma cell line 1c1c7 (21); and (iii) *in vitro* studies indicate that lysosomal proteases can mediate cytochrome *c* release from intact mitochondria through Bid cleavage, which is severely impaired in cytosolic extracts from Bid-deficient animals (9). Our results confirm these suggestions, identify Bid as a substrate of papain-like cathepsins, eliminate the aspartic protease cathepsin D as the cleaving enzyme, and indicate that caspases are not responsible for Bid cleavage in this model of lysosome-mediated apoptosis. Thus, we hypothesize that apoptosis triggered in HeLa cells by selective disruption of lysosomes proceeds through Bid cleavage mediated by papain-like lysosomal cysteine protease.

*In vitro*, Bid was cleaved by cathepsins B, H, L, S, and K and by the lysosomal extract at neutral pH, which supports other studies indicating that cathepsins can efficiently cleave their substrates at neutral pH (reviewed in Ref. 10). Intriguingly, although cathepsin H normally acts as an aminopeptidase (18, 54), in this context it acts as an endopeptidase. Thus, Bid represents one of the few identified protein substrates for which cathepsin H acts as an endopeptidase. The failure of cathepsins X and C to cleave Bid is probably related to their inability to cleave substrates at neutral pH and not to their exclusively exopeptidase activity. Cathepsin X is known to cleave substrates only in a very narrow pH range (pH 5.0–6.0; Ref. 28), whereas cathepsin C at neutral pH does not act as a protease but rather as an transpeptidase (55). The inability of cathepsin D to cleave Bid at neutral pH falls into the same category and is consistent with our inhibition studies demonstrating that pepstatin provided only minor protection against Bid cleavage even at high concentrations (Fig. 3*A*). It can be therefore surmised that apoptosis triggered by cathepsin D (14, 56) proceeds through pathways not involving Bid cleavage.

The multiple cleavage sites observed for most of the cathepsins suggest that their proteolysis of Bid differs from the controlled cleavage observed by caspase-8 and granzyme B by being a sequential event with the major sites attacked first, which is consistent with the rather nonselective protein degradation by cathepsins in lysosomes (18, 19). However, the caspases and granzyme B have a much more stringent substrate specificity than the cathepsins (57–59), and so multiple cathepsin-mediated cleavages are to be expected. Identification of Arg$^{65}$ and Arg$^{71}$ as the major cleavage sites is in complete agreement with substrate specificity of papain-like proteases, where positively charged residues are readily accepted in the P1 position (60). The other sites are not the most typical for papain-like cathepsins. However, the major specificity-determining sites in papain-like cysteine proteases are the P2 and P1′ sites, where hydrophobic residues are generally preferred (60), explaining cleavage at the other sites. Multiple cleavage appears not to be a problem for driving the apoptotic potential of Bid, because cytochrome *c* was readily released from mitochondria at both higher and lower cathepsin concentration, and all of the tBid variants were capable of inducing apoptosis in HEK 293 cells (Fig. 6). Nevertheless, under more physiological conditions with moderate lysosome damage, Bid degradation is relatively slow, because discrete Bid bands were observed in HeLa cells (Fig. 2). This attests to the proteolytically sensitive flexible loop that joins the second and third helices of Bid (Fig. 5).

Based on these results the following model is proposed for cathepsin-triggered apoptosis (Fig. 7). Various insults, among them oxidative stress and cytotoxic agents (61–63), can destabilize the lysosomal membrane, thereby releasing lysosomal proteases into the cytosol rather nonselectively, the major criterion being the size (<70 kDa; Ref. 56). Once in the cytosol the cathepsins can cleave Bid, followed by tBid-induced cytochrome *c* release, although the mechanism of the latter is not clear. Cytochrome *c* release would be expected to facilitate apoptosome formation and subsequent caspase-9 and executioner caspase-3, -7, and -6 activation. Most likely the pathway is regulated by the concentration of endogenous inhibitors, primarily stefin B for the cathepsins and XIAP (X-linked inhibitor of apoptosis) for the caspases, which provide a regulatory threshold to prevent accidental apoptosis triggering (42).

Cathepsins are redundant in this mechanism, but major roles are probably played by cathepsins B, L, and H, which are ubiquitous and most abundant among the cathepsins (18, 45). In particular, cathepsin B might be important by being sub-

stantially more stable than cathepsin L at neutral pH (64) and by being inhibited by stefin B, the major endogenous intracellular inhibitor of papain-like cathepsins, with the lowest affinity (42, 65). Because of its high stability at neutral pH (10) and moderate inhibition by stefin B, cathepsin H may also be important, although its signature proteolytic cleavage of Bid at Arg$^{71}$ was not observed with lysosomal extracts. However, cathepsin H might be responsible for Bid cleavage in hepatoma 1c1c7 cells, because it can be only very poorly inhibited by Z-FA-fmk, which was used in the study (21). A role for the other cathepsins (S, K, F, and V) cannot be completely discounted, especially in the tissues where they can be found at somewhat higher concentrations. Therefore, deleting a single cathepsin would probably have no effect in cell death scenarios where papain-like cathepsins have a major role and may thus be restricted to a limited number of examples, such as cathepsin B deficiency in EPM-1 (51) or in tumor necrosis factor-triggered hepatocyte apoptosis (15, 16).

It is far from clear that lysosomes play a role in physiologically programmed apoptosis or the normal response to apoptotic triggers. However, apoptosis in various cell lines triggered by various lysosomotropic reagents and oxidative stress (66) suggests a route for pathologic activation of the apoptotic machinery. Indeed, the sensitivity of lysosomes toward oxidative stress could predispose them to a number of pathological conditions, such as certain forms of neurodegeneration where oxidation plays a role (67–69).

Bid cleavage by papain-like cathepsins is not universal and cannot be applied to any apoptosis model triggered by lysosome destabilization. This applies not only to models where cathepsin D was shown to have a major role (11, 14, 56), but also to models where proteases have not been identified yet (10, 11, 63, 70). Indeed, some models where papain-like cathepsins are apparently the major players but a role for Bid is less apparent must have another explanation, such as cathepsin B-mediated apoptosis in cancer cells (17) and cathepsin B-mediated cerebellar apoptosis in stefin B-deficient animals (51, 71).

In conclusion, apoptosis triggered by selective lysosome disruption was analyzed at the molecular level in a cellular system, revealing Bid cleavage by papain-like cathepsins independent of caspase activation. Furthermore, several cathepsins cleaved Bid and facilitated cytochrome *c* release from mitochondria in the presence of Bid *in vitro*, indicating their redundant role. However, lysosomal proteases can also trigger apoptosis by pathways other than Bid cleavage, which remain to be elucidated at the molecular level.

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**Selective Disruption of Lysosomes in HeLa
Cells Triggers Apoptosis Mediated by
Cleavage of Bid by Multiple Papain-like
Lysosomal Cathepsins**

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