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Ionomycin-activated Calpain Triggers Apoptosis

A PROBABLE ROLE FOR Bcl-2 FAMILY MEMBERS*

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Ubiquitous calpains (μ- and m-calpain) have been repeatedly implicated in apoptosis, but the underlying mechanism(s) remain(s) to be elucidated. We examined ionomycin-induced cell death in LCLC 103H cells, derived from a human large cell lung carcinoma. We detected hallmarks of apoptosis such as membrane blebbing, nuclear condensation, DNA ladder formation, caspase activation, and poly-(ADP-ribose)polymerase cleavage. Apoptosis was prevented by preincubation of the cells with the calpain inhibitor acetyl-calpastatin 27-peptide and the caspase inhibitor Z-DEVD-fmk, implicating both the calpains and caspases in the apoptotic process. The apoptotic events correlated in a calpastatin-inhibitable manner with Bid and Bcl-2 decrease and with activation of caspases-9, -3, and -7. *In vitro* both ubiquitous calpains cleaved recombinant Bcl-2, Bid, and Bcl-x_L at single sites truncating their N-terminal regions. Binding studies revealed diminished interactions of calpain-truncated Bcl-2 and Bid with immobilized intact Bcl-2 family proteins. Moreover, calpain-cleaved Bcl-2 and Bid induced cytochrome *c* release from isolated mitochondria. We conclude that ionomycin-induced calpain activation promotes decrease of Bcl-2 proteins thereby triggering the intrinsic apoptotic pathway.

Studies on a number of cell types, such as neurons that die during formation of the nervous system and lymphocytes that die during receptor repertoire selection in adults, have shown that cells can activate built-in suicide mechanisms (1). Programmed cell death, or apoptosis, is beneficial during embryonic development and adult life but its dysregulation accompanies the pathogenesis of many diseases (reviewed in Ref. 2). Inappropriate increase in apoptotic cell death has been reported in wasting diseases such as AIDS, neurodegenerative disorders, and ischemic injury, whereas decreases in cell death contribute to proliferative autoimmune diseases and tumorigenesis. A precise description of cell death pathways and their regulation is therefore critical to understand cell death-associated diseases and to develop therapeutic strategies.

Proteolytic enzymes of the caspase family play a central role in initiating and sustaining the biochemical events that result in apoptotic cell death (3). In some forms of apoptosis, the extrinsic apoptotic pathway is initiated by activation of the apical caspase-8 following death receptor ligation (4). In other forms, cellular stress leads to activation of the intrinsic apoptotic pathway initiated by the apical caspase-9 (5). These pathways converge upon activation of the executioner caspases-3 and -7. Superimposed on this minimal scheme is an "integration model" in which both upstream and downstream caspases as well as other proteases cooperate to regulate apoptosis in a cell-specific manner (reviewed in Ref. 6).

The intrinsic pathway is triggered by cytochrome *c* release from mitochondria (7). The mechanism by which cytochrome *c* crosses the mitochondrial outer membrane is not yet known, but the opposing actions of a group of intracellular proteins, the Bcl-2 family proteins, critically regulate this process (8). The Bcl-2 family has been divided into three groups based on structural similarities and functional criteria (reviewed in Ref. 9). Group I consists of anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, which possess four short conserved Bcl-2 homology (BH)[1] domains (BH1–BH4). Group II members, exemplified by the pro-apoptotic proteins Bax and Bak, have a similar overall structure, containing all but the BH4 domains. Group III proteins consist of a large and diverse collection of proteins, exemplified by the pro-apoptotic Bid and Bik, whose common feature is the presence of the BH3 domain.

The function of calcium in apoptosis is a complex subject involving interplay between many systems, such as the sphingomyelin signaling pathway, the redox system, the stress-activated protein kinase cascade, and the calcium signaling path-

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[1] The abbreviations used are: BH, Bcl-2 homology; AC27P, acetyl-calpastatin 27-peptide; Ac-DEVD-amc, acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; μCP, μ-calpain; mCP, m-calpain; ECFP, enhanced cyan fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; PARP, poly-(ADP-ribose)polymerase; Suc-LLVY-, succinyl-Leu-Leu-Val-Tyr-; biotin-VAD-fmk, biotin-Val-Ala-Asp-fluoromethyl ketone; Z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Z-LLY-CHN_2, benzyl-oxycarbonyl-Leu-Leu-Tyr-diazomethane; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine]; FACS, fluorescence-activated cell sorter.

way (reviewed in Ref. 10). Regarding this, it has been reported that Bcl-2 family proteins may exert some of their actions by interfering with calcium dynamics of mitochondria and endoplasmic reticulum (10).

Increase of intracellular Ca^{2+} level induces apoptosis in thymocytes (11), hypocampal neurons (12), and various cancer cells via activation of calcium-dependent enzymes (*e.g.* Ref. 13). Addition of a calcium ionophore such as A23187 rapidly leads to activation of the ubiquitous calpains (14). Calpains are intracellular calcium-dependent cysteine endopeptidases but are otherwise structurally unrelated to caspases. The calpain family members can be classified as typical calpains, which are further divided into ubiquitous and tissue-specific calpains, and atypical calpains (reviewed in Ref. 15). Ubiquitous calpains occur in two isoforms, μ-calpain (μCP) and m-calpain (mCP) requiring micromolar or millimolar calcium concentrations for their activation *in vitro*, respectively. These heterodimeric enzymes consist of a small regulatory subunit (30 kDa) and a large catalytic subunit (80 kDa). In this work the term "calpains" refers to endogenous ubiquitous calpains.

The physiological roles of calpains are largely unknown, although their ubiquitous and constitutive expression strongly suggests essential and basic functions *in vivo*. In particular, connections to apoptotic processes have been suggested. For instance, calpains may be involved, in synergy with caspases, in the down-regulation of inositol 1,4,5-trisphosphate receptor subtypes during tumor necrosis factor-α-induced apoptosis of human T lymphoma cells (16). Cross-talk between calpains and caspases has been reported in the activation of caspase-12 (17), during etoposide-induced apoptosis in T cells (18), and during apoptosis of neuronal cells induced by a prion protein fragment (19). Synergistic involvement of calpain and the proteasome in apoptosis of human neutrophils has also been reported (20). Other authors (21) have suggested that calpains are activated via caspase-mediated cleavage of calpastatin during initiation of apoptotic execution. Finally, the transcription factor p53 is cleaved by ubiquitous calpains, suggesting a link between calpain activation and p53-dependent apoptosis (22).

Here we report that the calcium ionophore ionomycin promotes apoptosis in LCLC 103H cells, a cell line derived from a human large cell lung carcinoma (23). These cells represent an excellent system to study the effects of calcium increase induced by ionomycin, helping us to elucidate the pathways responsible for calcium-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Human μCP was isolated from erythrocytes according to Ref. 24; mCP was supplied by Calbiochem. Recombinant Bcl-2 proteins were purified as described elsewhere. Actin was from ICN Biomedicals. The following antibodies were used: anti-mouse Bax, anti-cytochrome *c*, anti-human Bid (BD PharMingen), anti-PARP (Biomol), anti-human Bcl-x_L (Kamiya Biomedical Co.), anti-human Bcl-2 (Dako), anti-caspase 8 (BD PharMingen), and anti-actin (Sigma). The fluorogenic substrates Ac-DEVD-amc and Suc-LLVY-amc were purchased from Bachem. AC27P was from Sigma; methylated Z-DEVD-fmk was from Alexis; and lactacystin was from Calbiochem. All other reagents were of the highest purity commercially available.

Cell Culture—For routine cell culture RPMI 1640 medium (Invitrogen) was supplemented with 10% fetal calf sera (Sigma), 0.6% L-glutamine. Transfection was carried out using the FuGENE™ 6 Reagent (Roche Molecular Biochemicals) according to the general protocol suggested by the manufacturer but applying the following reagent ratios: 1 μg of DNA, 1.5 μl of FuGENE™ 6 Reagent, 33 μl of serum-free RMPI 1640. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. LCLC 103H cells (DSMZ ACC 384), a clone stably expressing the chimeric protein histone H2A.i-ECFP, and LCLC 103H cells transiently overexpressing GFP (pNT-GFP, Invitrogen) were used in this study. When being subcultured, cells were lifted using a trypsin/EDTA solution (Invitrogen).

Monitoring of Nuclear and Cellular Changes—Cells were cultured on inner diameter 4.2-cm coverslips (Langenbrick) and treated with the indicated insult. For fluorescence microscopy, coverslips were mounted on POC chambers (PeCon), kept at 34–36 °C and 5% CO_2 in the microscope (Axiovert S100 TV, Zeiss) equipped with objectives, Fluor 40/1.3 oil Ph2 and Neofluar 63/1.25 oil Ph3, filter wheels, and shutters (Ludl), Orca 4742-95 CCD camera (Hamamatsu), and controlled by OpenLab software (Improvision). Filter systems were from Chroma Technologies; GFP excitation 490/20 nm, beam splitter 505 nm, emission 535/40 nm; ECFP excitation 470/30 nm, beam splitter 470/50 nm + 565/80 nm, emission 560/30 nm. For presentation, images were contrast-enhanced and "false-colorized" with the OpenLab software.

Apoptotic DNA Ladder Detection—Total DNA was isolated (25), separated on 2% agarose gels, and stained with ethidium bromide.

Isolation of Cytoplasmic Proteins—Cytoplasmic extracts were prepared by Dounce homogenization and differential centrifugation as described previously (26). Samples were mixed with sample buffer, heated for 2–5 min at 95 °C, and electrophoretically resolved on SDS-Tricine (16%) gels. Finally, samples were transferred to nitrocellulose membranes (Schleichler & Schuell) and probed against the indicated antibodies. Antibody complexes were detected with anti-mouse IgG or anti-rabbit horseradish peroxidase-linked IgG from New England Biolabs using ECL (Amersham Biosciences). Protein content was assayed by the bicinchoninic acid method (27).

Affinity Labeling—Aliquots of cytosolic proteins were incubated for 30 min at 37 °C with 2 μM biotin-VAD-fmk (Enzyme System Products). After incubation, extracts were diluted to 0.5 μg/μl in 4-fold concentrated SDS-Tricine sample buffer. Electrophoresed samples were transferred to nitrocellulose membranes, probed with peroxidase-labeled streptavidin, and visualized by Super Signal West Pico chemiluminescent substrate (Pierce).

Calpainolysis of Bcl-2 Proteins in Vitro—Recombinant Bcl-2 (4.2 μM), Bcl-x_L (4.5 μM), Bid (4.8 μM), and actin (2.4 μM) were incubated with purified μCP or mCP for 1 h at room temperature at a 1:100 enzyme/substrate molar ratio in calpain assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5, 20 mM dithiothreitol, 5 mM $CaCl_2$). Reactions were stopped by addition of 10 mM EDTA and iodoacetamide, separated on SDS-Tricine (16%) gels, and either visualized by Coomassie Blue staining or transferred to nitrocellulose or polyvinylidene difluoride membranes. Samples for Biacore experiments were prepared with a 1:10 molar calpain/protein ratio. The purified recombinant proteins and calpainolytic products were subjected to N-terminal amino acid sequencing on a PROCISE™ protein sequencer (Applied Biosystems Inc.).

Cytochrome c Release in Vitro—Mitochondria were isolated from rat heart according to the procedure described previously (28). Mitochondrial protein content was estimated by preincubating an aliquot of this preparation with 5% Triton X-100, and thereby the released cytochrome *c* was assumed to represent the total mitochondrial cytochrome *c* content. To analyze cytochrome *c*-releasing activity of intact and calpain-cleaved Bcl-2 proteins, aliquots of rat mitochondria corresponding to 15 μg of protein were incubated with 10 μl of the following samples prepared according to the calpainolysis protocol explained above: (i) Bcl-2 protein preincubated with Ca^{2+}, (ii) μCP preincubated with Ca^{2+}, and (iii) Bcl-2 protein preincubated with μCP and Ca^{2+}, according to Ref. 29. The final protein concentrations are as follows: Bcl-2, 1.0 nM; Bcl-x_L, 1.8 nM; and Bid, 10.1 nM. Gels were transferred to nitrocellulose membranes, probed with anti-cytochrome *c* antibody, and analyzed by densitometry (Scion Image).

Biomolecular Interaction Analysis of Bcl-2 Proteins—Interactions between Bcl-2 family proteins and their calpainolytic products were analyzed in a Biacore 2000 instrument (Biacore AB) essentially as described previously (30). Bcl-x_L, Bax, Bcl-2, and Bid were immobilized on CM5 sensor chips via amine coupling (2000–4000 resonance units). Calpainolytic reaction mixtures of Bid, Bcl-2, and Bcl-x_L were prepared as described above, except that after addition of EDTA the pH was adjusted to 6.0 (Bcl-2) or 7.5 (Bid, Bcl-x_L) and diluted 8-fold with running buffer (50 mM acetate, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 4.0 for Bcl-x_L and Bid, 50 mM phosphate, 3.4 mM EDTA, 0.005% Tween 20, pH 6.4 for Bcl-2). Analytes (70 μl at 0.5–2.0 μM) were injected at a flow rate of 10 μl/min. Sensorgrams of ethanolamine blanks were used for subtraction of changes in the bulk refractive index. Between sample injections, the sensor chips were regenerated with 50 mM phosphate (pH 6.8) and 4 M guanidine HCl. The sensorgrams were analyzed using the BIAevaluation 3.1 software.

Protease Activity in Living Cells—Calpain- or caspase-like activities in whole cells were measured with the fluorogenic substrates Suc-LLVY-amc (160 μM) or Ac-DEVD-amc (200 μM), respectively. The sub-

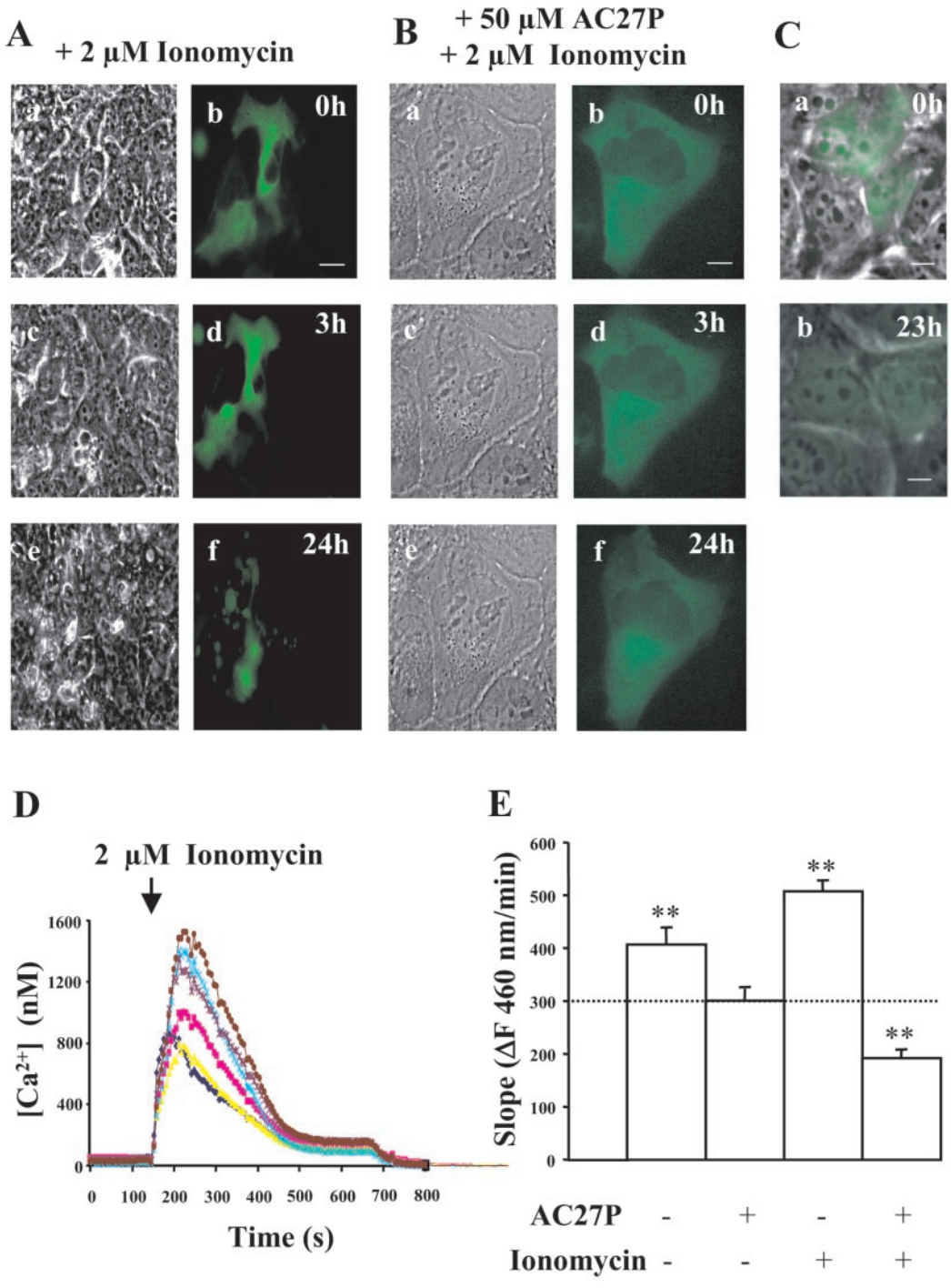

FIG. 1. **Induction of cell death and calpain activation in LCLC 103H cells by ionomycin.** *A* and *B* show the time course of cell death after addition of ionomycin to LCLC 103H cells overexpressing GFP either without (*A*) or after preincubation with 50 μM AC27P (*B*). *Left* (*a*, *c*, and *e*) and *right lanes* (*b*, *d*, and *f*) represent phase contrast and fluorescence images, respectively. *C*, ectopic expression of GFP does not trigger apoptosis in LCLC 103H cells. Superimposition of phase contrast and fluorescence images before (*a*) and 23 h after addition of ionomycin (*b*). *A*, *bars* represent 50 μm; *B*, 5 μm; *C*, 50 (*a*) and 25 μm (*b*). *D*, increase of intracellular calcium concentration upon ionomycin addition to LCLC 103H cells. Cells were loaded with Fura-2 for 30 min and treated with 2 μM ionomycin as explained under "Experimental Procedures." Calcium concentration changes monitored in six different cells are represented in *dark* or *light blue*, *yellow*, *pink*, *violet*, and *brown*. *E*, AC27P-inhibitable Suc-LLVY-amc-hydrolyzing activity increases after addition of 2 μM ionomycin to LCLC 103H cells. Cleavage rates of the fluorogenic substrate Suc-LLVY-amc (slopes of the progress curves, Δ*F* 460 nm/min, in arbitrary units) were calculated from amc-fluorescence recorded between 5 and 170 min (linear phase of substrate cleavage) as the mean of three experiments (S.D. <10%, indicated by *bars*). **, differences to activity after preincubation with AC27P (*2nd column*, *dashed line*) statistically significant ($p < 0.01$).

strates ionomycin, etoposide, and AC27P were mixed in the appropriate HEPES-buffered serum-free growth media. LCLC 103H cells were plated on 24-well plates (10^5 cells/well) and preincubated with the substrate for 30 min at 37 °C in a humidified 5% CO_2 incubator. Substrate hydrolysis at 37 °C was monitored using a fluorescence reading system (Fluoroskan ascent) set to 355 ± 20 nm for excitation and 460 ± 20 nm for emission. Fluorescence readings were collected every 5 min (up to 400 min), before and after addition of ionomycin, and after preincubation of the cells for 1 h at 37 °C with 50 μM AC27P or 20 μM of Z-DEVD-fmk.

FACS Analysis of Apoptotic Cells—Cell viability was assessed by flow cytometry simultaneously monitoring annexin V binding and propidium iodide uptake. LCLC 103H cells were plated on inner diameter 10-cm culture plates and treated at 37 °C with the indicated inhibitor/

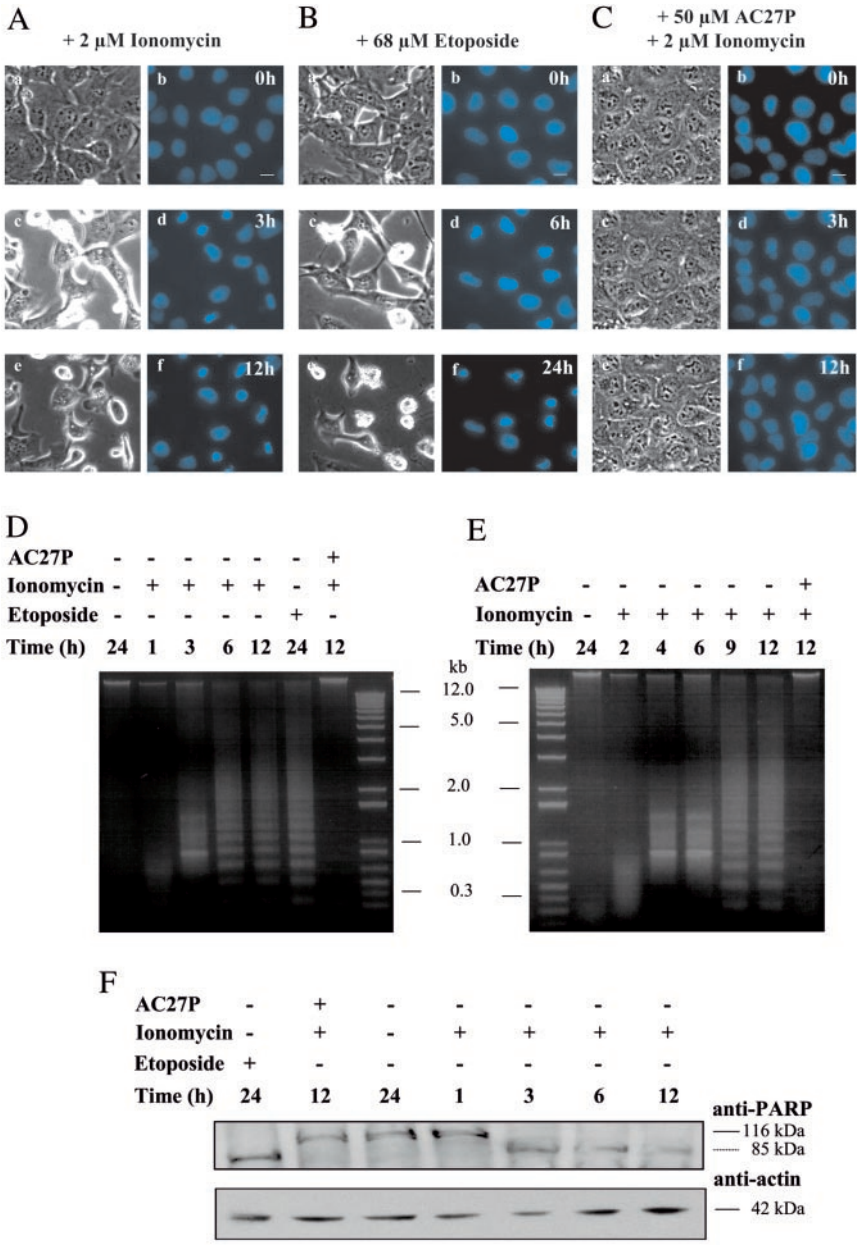

FIG. 2. **Hallmarks of apoptosis in ionomycin-treated LCLC 103H cells.** Nuclear changes in LCLC 103H cells stably overexpressing H2A.i-ECFP after treatment with 2 μM ionomycin (*A*), 68 μM etoposide (*B*), or 2 μM ionomycin after preincubation with 50 μM AC27P (*C*). *Left* (*a*, *c*, and *e*) and *right lanes* (*b*, *d*, and *f*) represent phase contrast and fluorescence images, respectively. *Bars* represent 25 μm. *D* and *E*, DNA ladder formation in ionomycin-treated LCLC 103H (*D*) and COS 7 cells (*E*). *F*, Western blots of cell lysates from LCLC 103H cells probed with anti-PARP and anti-actin antibodies. Endogenous intact proteins and cleaved PARP (85 kDa) are indicated by *solid* and *dashed lines*, respectively.

insult, resuspended in 2 ml of annexin V binding buffer, and finally treated with annexin V-fluorescein and propidium iodide (9:1) for 5 min at room temperature. Fluorescence of 20000 cells was measured with a FACS-Calibur Sort (BD PharMingen) through a 530/30 bandpass filter to monitor annexin-fluorescein-phosphatidylserine binding and through a 585/42 filter to monitor propidium iodide uptake.

Measurement of Free Calcium Concentrations—Free calcium concentrations in LCLC 103H cells were determined before and after adding 2 μM ionomycin. These measurements and the calibration of Fura-2 fluorescence was performed according to standard protocols (31). Briefly, cells were loaded for 1 h with 5 μM Fura-2 AM diluted in Fluoronic^R F-127. Coverslips were rinsed with a solution containing 20 mM HEPES, 5.6 mM glucose, 137 mM NaCl, 0.8 mM KCl, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2 mM EDTA, pH 7.4, placed in an imaging chamber, and mounted in a platform at 37 °C on the stage of a Nikon Diaphot. Fura-2 was excited at alternating wavelengths of 340- and 380-nm using a 75-watt xenon light source and a filter wheel (Ludl). Emitted wavelengths passed through a 510-nm filter cube set before detection by an enhanced CCD camera. Saturating calcium concentrations were measured after rinsing and preincubation of the cells with a solution of 10 mM $CaCl_2$ in 20 mM HEPES, pH 7.4, containing 5.6 mM glucose, 137 mM NaCl, 0.8 mM KCl, 1.0 mM $MgCl_2$, 2 mM EDTA, and two subsequent additions of 2 μM ionomycin with 5-min interval between them. After that, absence of calcium was measured by adding 2 ml of 80 mM EGTA, pH 8.0. Data were stored and processed using the IonWizard software.

RESULTS

Ionomycin Induces Apoptosis in LCLC 103H Cells—In the course of calpain localization studies, we observed that LCLC 103H cells overexpressing the catalytic subunit of μ-calpain rapidly underwent cell death after treatment with ionomycin (not shown). To monitor this process in cells not overexpressing calpain, we labeled LCLC 103H cells transiently with GFP or stably with a histone-ECFP chimera. The first signs of ionomycin-induced cell death were detected 3 h after addition of the ionophore. We observed typical hallmarks of apoptosis, including cell blebbing and fragmentation, which were prevented for 24 h by preincubation with the selective calpain inhibitor AC27P (Fig. 1, *A* and *B*). Untreated cells overexpressing GFP (Fig. 1*C*) or the chimeric-ECFP protein (not shown) were not impaired in their vitality, ruling out that ectopic expression of either protein is cytotoxic for this cell line.

Addition of 2 μM ionomycin to LCLC 103H cells caused an

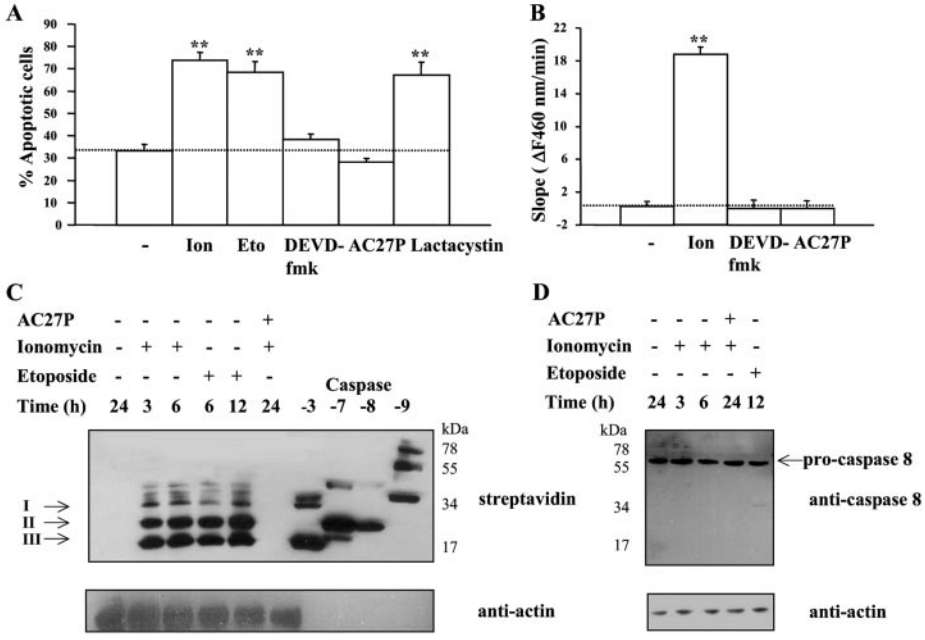

FIG. 3. **Caspase activation in ionomycin-induced apoptosis of LCLC 103H cells.** *A*, effect of calpain and caspase inhibitors on ionomycin-induced apoptosis. Cells treated with the indicated insults/inhibitors had annexin V-fluorescein and propidium iodide added before being counted and sorted by FACS as described under "Experimental Procedures." % apoptotic cells represents the percentage of cells positive for phosphatidylserine externalization and negative for propidium iodide related to the total amount of cells. *B*, caspase activity measured in LCLC 103H cells after 30 min of incubation at 37 °C with the fluorogenic substrate Ac-DEVD-amc (Δ*F*460 nm/min, in arbitrary units). −, spontaneous activity; *Ion*, activity after addition of 2 μM ionomycin; *Eto*, activity after treatment with 68 μM etoposide; *DEVD-fmk*, *AC27P*, and *lactacystin*, activities in cells preincubated with the corresponding inhibitors before addition of 2 μM ionomycin. All experiments were performed in triplicate; S.D. is indicated by *bars*. **, difference to non-treated cells (−, *dashed line*) statistically significant ($p < 0.01$). *C*, presence of active caspases-3, -7, and -9 in the cytosol of apoptotic cells. Cytosolic proteins were affinity-labeled with biotin-VAD-fmk. Biotinylated recombinant caspases 3, -7, -8, and -9 were applied as standards (see "Results" for explanation of *bands I–III*). Notice that active species are absent in cytosolic lysates of cells pretreated with AC27P. *D*, Western blot using an anti-caspase-8 antibody showing that pro-caspase-8 is not activated in ionomycin-induced apoptosis.

instantaneous increase in intracellular Ca^{2+} concentration from 50 to 180 nM (Fig. 1*D*). Remarkably, calcium concentrations were raised transiently to 0.8–1.5 μM. Concomitantly, calpains were activated, as reflected by almost a double increase of Suc-LLVY-amc cleavage inhibited by AC27P (Fig. 1*E*). In addition, nuclear condensation was detected in the histone-ECFP-labeled cells 3 h after addition of ionomycin (Fig. 2*A*), and it was inhibited by preincubation with AC27P (Fig. 2*C*). DNA and protein analysis in ionomycin-treated cultures revealed DNA fragmentation (Fig. 2*D*) and PARP cleavage to an 85-kDa fragment typical of caspase-mediated apoptosis (Fig. 2*F*). The same observations were made in etoposide-treated LCLC 103H cells (Fig. 2*B*) and in COS 7 cells after ionomycin addition (Fig. 2*E*), suggesting an apoptotic process, although its onset was delayed in comparison to ionomycin-treated LCLC 103H cells. These findings demonstrate that ionomycin induces apoptosis in these cells in a calpain-dependent manner.

Caspase Involvement in Ionomycin-induced Apoptosis—Flow cytometric analysis of fluorescein-labeled annexin V binding and propidium iodide uptake were used to quantify early apoptotic cells. Three hours after addition of ionomycin, the percentage of early apoptotic cells approximately doubles (Fig. 3*A*). Necrosis could be detected in ~1–5% of the ionomycin-treated cells (not shown) as supported by simultaneously positive fluorescein labeling and propidium iodide uptake. Apoptosis of LCLC 103H cells was inhibited by preincubation with AC27P or the caspase inhibitor Z-DEVD-fmk. In contrast, apoptosis could not be prevented by preincubation of cells with the proteasome inhibitor lactacystin. Caspase activation in whole cells was followed by monitoring the increase in activity against Ac-DEVD-amc following ionomycin treatment. In addition, we evaluated the effect of preincubating the cells with either AC27P or Z-DEVD-fmk (Fig. 3*B*). Cleavage of Ac-DEVD-

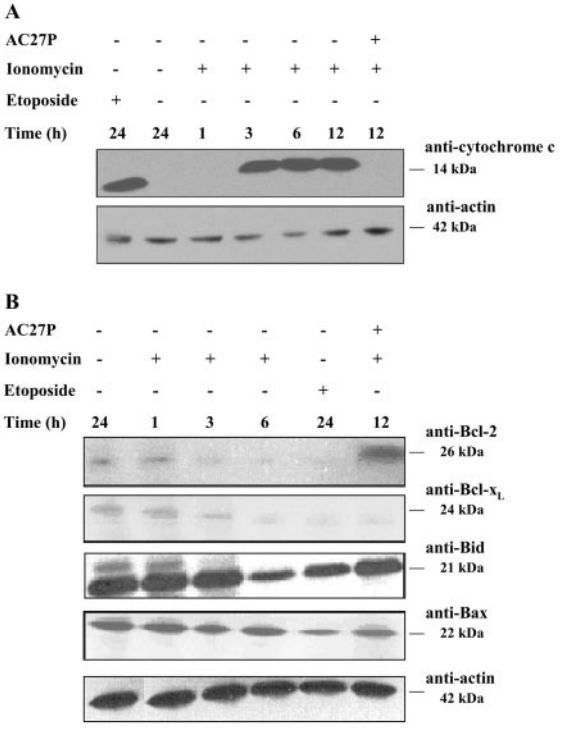

FIG. 4. **Ionomycin-induced death of LCLC 103H cells is accompanied by cytochrome *c* release and changes in the concentrations of Bcl-2 proteins.** *A*, ionomycin-induced cytochrome *c* release *in vivo*. Western blots for cytochrome *c* and actin detection. *B*, processing of endogenous Bcl-2, Bcl-x_L, Bid, Bax, and actin *in vivo*. Western blots for Bcl-2, Bcl-x_L, Bid, Bax, and actin (indicated by *solid lines*). Actin was neither processed during ionomycin-induced apoptosis nor in etoposide-treated cells.

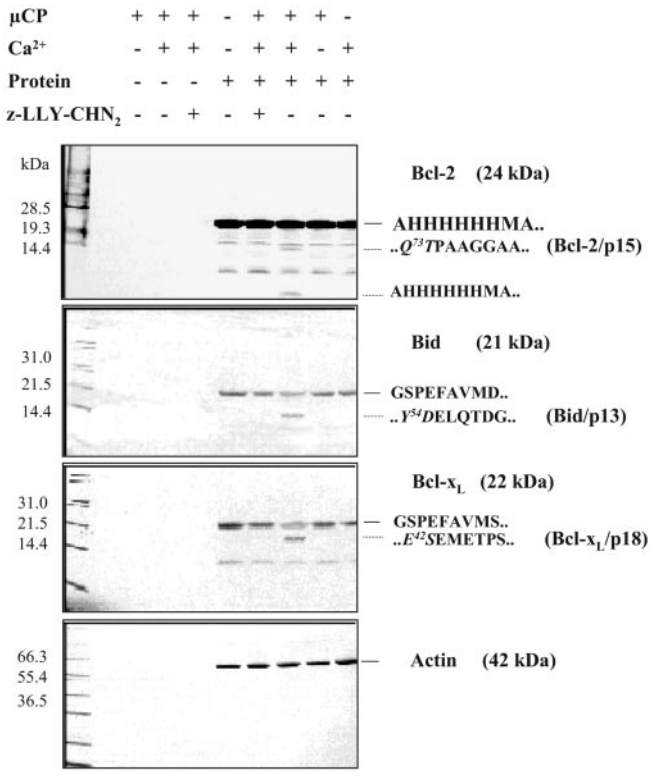

FIG. 5. **Calpainolysis of Bcl-2 family proteins *in vitro*.** SDS-Tricine (16%) gels showing the effects of μ-calpain on Bcl-2, Bid, Bcl-x_L, and actin. The intact recombinant proteins (*solid lines*) and their cleavage products (Bcl-2/p15, Bid/p13, and Bcl-x_L/p18; *dashed lines*) were identified by N-terminal sequencing (*right margin*). Results are representative of five replicas. Notice that actin was not cleaved by calpain *in vitro*.

amc predominantly reflects the activity of executioner caspase-3, although other caspases may also contribute to the increase in activity (32). Caspase activation occurs 3 h after ionomycin treatment and is inhibited to a similar extent by AC27P and by Z-DEVD-fmk.

To delineate more completely the spectrum of caspases activated in LCLC 103H cells, cytosolic extracts were incubated with the affinity label biotin-VAD-fmk. Three discrete bands termed I (~35 kDa), II (~25 kDa), and III (~17 kDa) were detected 3 h after the addition of 2 μM ionomycin (Fig. 3*C*). The use of antisera to detect cleavage of caspases-3, -7, and -9 is indicative of activation but is not definitive because all of the active enzymes are expected to be complexed *in vivo* by members of the IAP family of caspase inhibitors (33, 34). Therefore, we sought to use a more direct assay for the activity of individual caspases. The mobility of the large subunits of active caspases detected via biotin labeling was compared with the mobility of the corresponding subunits of recombinant human caspases (Fig. 3*C*). In SDS-Tricine gels, the lower band III migrates as the large subunit of the executioner caspase-3, with band II migrating similar to both recombinant caspases-7 and -8. Finally, band I is consistent with alternatively processed caspase-9 (equivalent to the 35-kDa band seen in Ref. 35). No active caspases were detected in cytosol from either untreated or AC27P-treated cells (Fig. 3*C*). Faint bands of higher molecular masses were also observed and either represent zymogens with slight activity (36) and/or the 37-kDa band seen for processed caspase-9 in Ref. 35. We could not detect caspase-8 processing and therefore no mature enzyme in any of the lysates (Fig. 3*D*). Taken together, these results indicate that ionomycin treatment is associated with activation of multiple caspases via the intrinsic (mitochondrial) pathway and furthermore strongly suggest that caspases-3, -7, and -9 are activated downstream of calpain after ionomycin treatment.

Ionomycin-induced Cytochrome c Release and Bcl-2 and Bid Decrease—During ionomycin-induced cell death we observed a marked release of cytochrome *c* from mitochondria 3 h after addition of the ionophore (Fig. 4*A*). Similar amounts of cytochrome *c* were released upon treatment of the cells with 68 μM etoposide. In contrast, no cytoplasmic cytochrome *c* was detected either in lysates from wild type LCLC 103H cells or in cells preincubated with AC27P before addition of ionomycin.

Next, we aimed to identify calpain substrates associated with the observed cell death processes. We had described previously (37) that caspases-3 and -9 are not directly activated by calpain, yet another report (38) concluded that calpains are able to activate procaspase-7 in apoptosis during clonal expansion of B cells. In our hands, low μ-calpain concentrations produced limited cleavage of recombinant procaspase-7, truncating its N terminus at positions Phe-36 and Met-45 (Phe-129 and Met-138 in the caspase-1 numbering system). However, these cleavages are irrelevant for generation of an active caspase, as revealed in the recent structures of human procaspase-7 (39, 40); and accordingly no activity against the caspase-7 substrate Ac-DEVD-amc was observed (not shown). At higher calpain concentrations both caspase-3 and -7 zymogens were completely degraded, and at no time was caspase activity detected (not shown).

In the absence of evidence for direct caspase processing, we explored whether calpains could cleave mitochondrial regulators of cytochrome *c* release, particularly members of the Bcl-2 family. Indeed, we noted differences in the concentrations of endogenous Bcl-2, Bcl-x_L, Bid, and Bax during ionomycin-induced cell death in LCLC 103H cells (Fig. 4*B*). Two hours after ionomycin treatment, the cytosolic amounts of Bcl-2 and Bcl-x_L decreased remarkably. Only 1 h later intact Bcl-2 could no longer be detected, and intact Bcl-x_L as well as Bid were diminished, whereas no Bax was degraded up to 3 h. Ionomycin-induced Bcl-2 and Bid decrease was prevented by preincubation with AC27P. Curiously, the intensity of the Bcl-2 band was highest in cells pretreated with AC27P, suggesting that this calpain inhibitor stimulates Bcl-2 expression (Fig. 4*B*), consistent with previous reports (41) that calpeptin pretreatment prior to A23187 prevented μ-calpain overexpression and decreased the Bax/Bcl-2 ratio.

Bcl-2 Family Proteins Are Calpain Substrates in Vitro—The results discussed above suggested that activated calpains may cleave several Bcl-2 proteins. To verify this hypothesis, recombinant Bcl-2 proteins were incubated with purified human m- and μ-calpain as described under "Experimental Procedures." Fig. 5 shows that μ-calpain cleaves recombinant Bcl-2, Bid, and Bcl-x_L at unique sites *in vitro* and generates the following truncated proteins: Bcl-2/p15, Bid/p13, and Bcl-x_L/p18 (named according to their approximate apparent molecular masses; similar results were obtained with mCP). Proteolysis depended on the presence of calpain and was prevented by the irreversible calpain inhibitor Z-LLY-CHN_2. Initial cleavage of Bcl-2, Bid, and Bcl-x_L was rapid, occurring 5 min after reaction start (not shown). The cleavage sites identified after incubation for 1 h are summarized in Table I and Fig. 6. After longer incubation times, additional cleavage sites were found in Bcl-2 behind His-94, Thr-96, and Arg-98. The cleavage products were not detected by the commercially available antibodies against the Bcl-2 family proteins used in this work (not shown).

Calpain-cleaved Bcl-2 and Bid Induced Cytochrome c Release from Isolated Mitochondria—Isolated mitochondria from rat cardiac muscle were incubated either with calpain alone, the

TABLE I
Summary of cleavage sites identified in Bcl-2 family proteins

Bcl-2 protein	Proteinase	Cleavage site	Ref.
Bcl-x_L	Caspase-3	Asp-61, Asp-76	63
Bcl-x_L	DEVD-cho-sensitive enzymes	Asp-61	61
Bcl-x_L	m-calpain	Ala-60	17
Bcl-x_L	μ- and m-calpain	Glu-42	This work
Bcl-2	HIV aspartic protease	Phe-112	64
Bcl-2	Caspase-3	Asp-34	56
Bcl-2	Z-VAD-fmk inhibitable protease	Asp-34	65
Bcl-2	μ- and m-calpain	Gln-73	This work
Bid	Caspase-8	Asp-59	66
Bid	Granzyme B	Asp-75	67
Bid	Lysosomal proteinases	Arg-65	29
Bid	Calpain	Gly-70	60
Bid	μ- and m-calpain	Tyr-54	This work

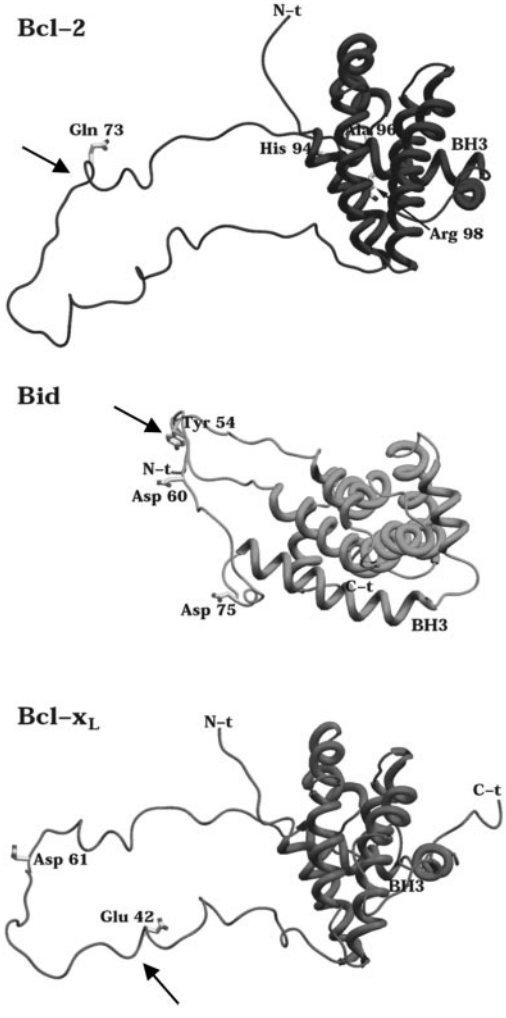

FIG. 6. **Ribbon plots of Bcl-2 family members indicating calpain cleavage sites.** Coordinates were taken from the deposited files, Protein Data Bank codes 1G5M (Bcl-2/Bcl-x_L chimera), 2BID (Bid), and 1MAZ (Bcl-x_L). A Bcl-2 model was constructed by replacing the artificial loop sequence of the Bcl-2/Bcl-xL chimera by the correct sequence found in human Bcl-2. The BH3 domains of the three proteins were superimposed using Turbo-FRODO, and the images were made with SETOR. All structures are shown at the same scale. *Arrows* point to the unique calpain cleavage sites found within the corresponding Bcl-2 family protein. Other cleavage sites are also indicated (see also Table I).

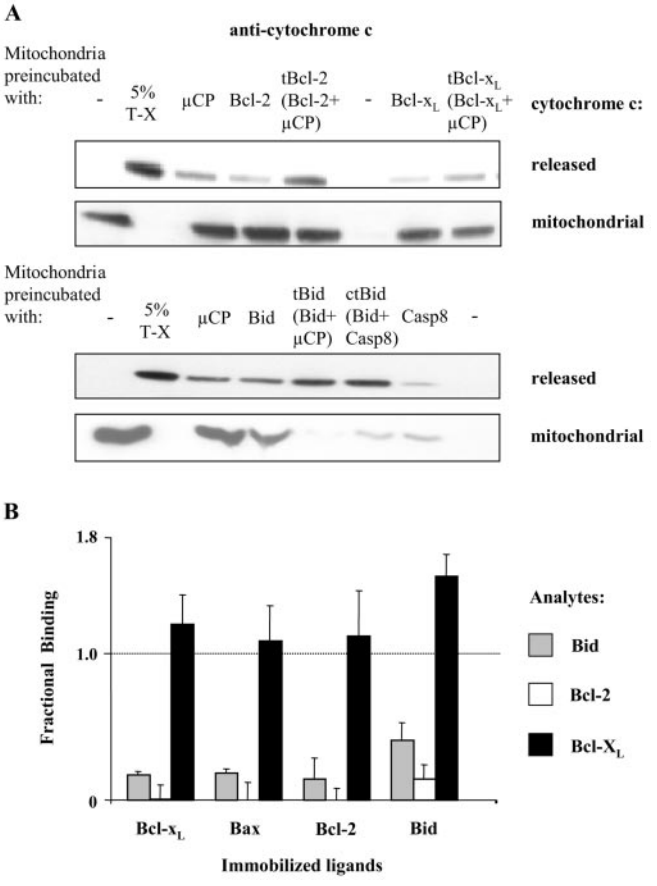

FIG. 7. **Effect of calpainolysis on Bcl-2 family proteins.** *A*, calpain-cleaved Bcl-2 and Bid induce cytochrome *c* release from isolated mitochondria. Mitochondria were incubated for 40 min at 30 °C either in the presence or absence of Bcl-2 proteins and calpain as explained under "Experimental Procedures." The supernatants (released cytochrome *c*) and pellets (mitochondrial cytochrome *c*) were resolved on SDS-Tricine (16%) gels followed by Western blotting using an anti-cytochrome *c* antibody. *Casp*, caspase. *B*, binding of calpain-cleaved Bid, Bcl-2, and Bcl-x_L to their immobilized intact counterparts is diminished. Biacore results obtained with two chips loaded with the immobilized ligands Bcl-x_L, Bax, Bcl-2, and Bid. *Fractional binding* surface plasmon response (resonance units) of the calpain-cleaved analytes Bid (*gray*), Bcl-2 (*white*), Bcl-x_L (*black*) expressed as a fraction of the response of the intact analytes, after subtraction of the response of calpain. All experiments were conducted in triplicate.

Bcl-2 proteins alone, or with the calpain-treated Bcl-2 proteins. Mitochondria incubated with either μ-calpain, caspase-8, Bcl-2, or Bid showed only very low cytochrome *c* release. In striking contrast, incubation of mitochondria with calpain-truncated Bcl-2 or Bid induced substantial or almost complete release of cytochrome *c* when compared with total mitochondrial cytochrome *c* content (Fig. 7*A*). Remarkably, calpain-truncated Bid released cytochrome *c* to a similar degree as caspase-8-cleaved Bid. In contrast, only minimal differences were detected in the amounts of cytochrome *c* released by calpain-truncated or intact Bcl-x_L (Fig. 7*A*).

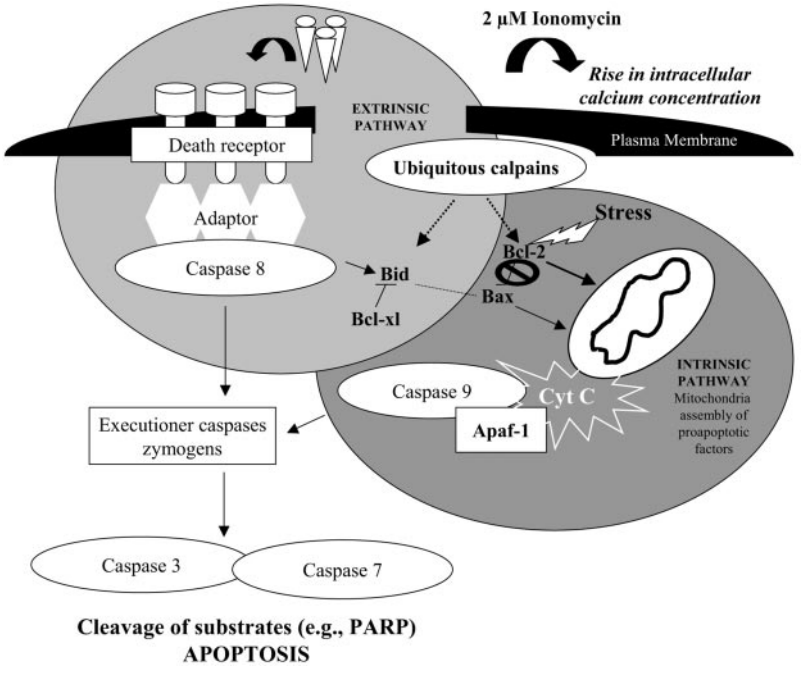

FIG. 8. **Hypothetic mechanism of calpain involvement in ionomycin-induced apoptosis of LCLC 103H cells.** The intrinsic pathway (*darker shading*) responds primarily to cellular stress (ionizing radiation, cytotoxic drugs, etc.) with mitochondria acting as important integrators. Ionomycin-activated calpains cleave Bcl-2 and Bid (indicated by *dashed arrows*), triggering cytochrome *c* release and hence the intrinsic apoptotic pathway. The "*no symbol*" stands for the diminished interactions between Bax and calpain-cleaved Bcl-2. *Apaf-1*, apoptotic protease activating factor 1.

Calpain Cleavage of Bcl-2 Family Proteins Affects Their Homo- and Heterophilic Interactions—Various Bcl-2 family proteins have been shown to influence mitochondrial integrity via poorly understood processes that involve both homo- and heterodimerization (42, 43). We applied surface plasmon resonance to monitor the interactions between intact and μ-calpain-cleaved Bcl-2 proteins *in vitro*. Intact Bcl-2 proteins were coupled to CM5 chips as described under "Experimental Procedures" and were used for Biacore binding studies. Cleavage sites within the calpain-cleaved Bcl-2 proteins were verified by N-terminal amino acid sequencing and correspond to those described above. Binding of calpain-treated Bcl-2 and Bid to all four intact Bcl-2 family proteins immobilized on chips was significantly reduced (Fig. 7*B*). In contrast, binding of calpain-cleaved Bcl-x_L to the Bcl-2 family proteins was unchanged or moderately enhanced.

DISCUSSION

Two cytosolic proteolytic systems are capable of producing limited cleavage of endogenous proteins, the caspases and the calpains. Although it is well established that several caspases are involved in, if not essential for, apoptosis (4, 44), the physiologic role of the calpains is much less clear (45). Ablation of the common non-catalytic 30-kDa subunit of μ- and m-calpain causes embryonic lethality in mice, pointing to essential functions of the enzymes (46). A number of studies have shown that calpain activation precedes cell death induced by different apoptotic stimuli in various cell systems (see *e.g.* Refs. 47 and 48). Therefore, we set out to dissect the involvement of ubiquitous calpain in a calcium-induced apoptotic pathway. The calpains are not able to produce the apoptotic phenotype on their own, but they presumably utilize the existing latent death machinery common to most nucleated metazoan cells. Previous work (37), confirmed here, has demonstrated that calpains are not able to activate caspases directly, so we sought other possible entry points into the apoptotic pathway. As crucial endogenous regulators of the intrinsic apoptotic pathway (43), Bcl-2 family proteins were likely targets.

Here we present evidence that ionomycin-activated calpain, as a consequence of calcium increase, triggers apoptosis in a large cell lung carcinoma cell line concomitantly with Bcl-2 and Bid decreases. Ionomycin-induced decreases in Bcl-2 and Bid levels correlated in a calpastatin-inhibitable manner with cytochrome *c* release, caspase activation, and PARP cleavage. Thus it appears that caspase activation proceeds through the intrinsic (mitochondrial) apoptotic pathway, with an involvement of Bcl-2 family members. Related to our results, a spatially and temporally regulated cascade has been proposed in a traumatic axonal injury model, in which calpain activation triggers intracellular changes involving release of cytochrome *c* from mitochondria, followed by activation of procaspase-3 (49).

Recently, it has been reported that calpain was activated in myocytes after superfusion with sodium-free solution when the intracellular calcium concentration was raised to 0.45 μM (50). Consistent with these results, we report here that calpain activation triggered by transient calcium concentration increases to 0.8–1.5 μM, induced by addition of 2 μM ionomycin.

Increase of AC27P-inhibitable Suc-LLVY-amc hydrolyzing activity after addition of ionomycin strongly suggests ionomycin-induced activation of calpain. Suc-LLVY-amc hydrolyzing activity that was not inhibited by AC27P could be a consequence of other proteolytic activities such as those of the lysosomal cathepsins B and L. Papain and the papain-like lysosomal cysteine proteinases are also able to cleave this fluorogenic substrate *in vitro* (51).[2] Interestingly, the Suc-LLVY-amc hydrolyzing activity decreases after preincubation with AC27P and further treatment with ionomycin (see Fig. 1*E*), suggesting the decrease of some proteolytic activities as a consequence of apoptosis induction.

The rules governing the specificity of calpains remain unclear. Substrate recognition seems to be determined by the overall three-dimensional structure rather than by particular sequence motifs. Most calpain substrates are proteolyzed in a restricted manner (52), suggesting that limited cleavage may modulate the function of these substrates. Consistent with this is our observation that ubiquitous calpains cleaved Bcl-2, Bcl-x_L, and Bid preferentially at single sites *in vitro*.

Bcl-2 proteins may regulate apoptosis either through protein-protein interactions or through formation of pores in lipid membranes (42). The members of the Bcl-2 family can het-

[2] I. Assfalg-Machleidt, S. Gil-Parrado, N. Schaschke, and W. Machleidt, manuscript in preparation.

erodimerize with other family members, for example Bcl-2 and Bax form heterodimers (53) that block apoptotic processes (54). Furthermore, x-ray crystallographic analysis has shown that the BH1/2/3 domains of Bcl-x_L form a surface-exposed hydrophobic groove (reviewed in Ref. 42), which docks the BH3 domains of pro-apoptotic binding partners such as Bax. We found that the intact proteins, Bcl-2 and Bid, but not their calpain-truncated forms, were able to homo- and heterodimerize to similar extents (see Fig. 7*B*). The sequential cleavages of Bcl-2 observed *in vitro* in this work point to significant conformational changes of Bcl-2 induced by cleavage at the primary calpain cleavage site, Gln-73, presumably causing dissociation of pro-apoptotic proteins such as Bax. Dissociation of Bax/Bcl-2 dimers would allow their translocation to the mitochondria (55), thus triggering the intrinsic apoptotic pathway.

Calpain-truncated Bcl-2 and Bid were able to release cytochrome *c* from isolated mitochondria. In contrast, we did not detect significant differences between cytochrome *c* release induced either by intact or calpain-truncated Bcl-x_L. It is noteworthy that removal of the N-terminal domain of Bcl-2, either by BH4 deletion or by caspase cleavage, converts Bcl-2 from an anti- to a pro-apoptotic molecule (56).

The cytosolic amounts of Bcl-2 and Bid decrease after ionomycin addition. These decreases are inhibited by AC27P suggesting (i) cleavage of the proteins by calpain or by calpain-induced proteases, (ii) the induction of a fast turnover of the proteins, and (iii) down-regulation of the Bcl-2 protein expression. By taking into account that this is not a long term process as expected for translation/modification events and, regarding the *in vitro* calpainolysis results (see Fig. 5), it is likely that calpain mediates ionomycin-induced apoptosis by direct cleavage of Bcl-2 proteins, such as Bcl-2 and Bid. We do not rule out that, once generated, these calpain-cleaved products could be likely targets of rapid turnover pathways like the proteasome as part of a survival attempt of the cell.

Also of relevance to this study, Bcl-2 may mediate some of its anti-apoptotic actions by modifying the way in which organelles such as the ER and mitochondria respond to variations in Ca^{2+} concentration (57). In normal cells, Bcl-2 may modify the Ca^{2+} properties of the ER (58) and the mitochondria (59) mainly by regulating the ER calcium concentration and thus preserving the intracellular Ca^{2+} balance. Therefore, it is conceivable that calpain could target Bcl-2 under certain stress conditions as part of a calcium regulatory or transduction pathway. A recent study (60) indicates that calcium influx after reperfusion following ischemia leads to calpain activation in rabbit hearts and that activated calpains cleave Bid to a fragment that promotes cytochrome *c* release. Cleavage of Bcl-2/Bid may in turn stimulate further calpain activation, resulting in a positive feedback activation loop.

Regardless of the mechanism Bcl-2 proteins use to induce apoptosis, proteolytic cleavage seems to be important. The vast majority of cleavage sites identified in Bcl-2 proteins map to highly flexible "bait loops" connecting BH4 and BH3 domains (Fig. 6 and Table I). Such regions are excellent targets for proteolysis, thus allowing cells to respond rapidly to adventitious and potentially damaging intracellular proteolytic activity by triggering the in-built apoptotic suicide program. Cleavage of the extended loop segments of Bcl-x_L (61) and Bcl-2 (56) by caspases has been reported previously to accelerate cytochrome *c* release *in vitro* or decrease viability. In the current work, cleavage of Bcl-x_L was also induced by ionomycin *in vivo* but was not prevented by preincubation of the cells with AC27P.

Caspases are activated after ionomycin addition and downstream of calpains, as AC27P inhibition of Ac-DEVD-amc cleavage activity suggests. We detected several polypeptide species by affinity labeling with biotin-VAD-fmk in LCLC 103H cells undergoing ionomycin-induced apoptosis. These polypeptides correspond to the large subunits of activated caspases-3, -7, and –9. The probable contribution of caspase-8 was ruled out because conversion of the 55-kDa zymogen was not observed. This provides the first direct evidence that multiple caspases are activated during apoptosis in LCLC 103H cells treated with ionomycin. Together with the results of *in vivo* and *in vitro* experiments with Bcl-2 proteins, this finding suggests a calpain-induced, cytochrome *c*-mediated activation of procaspase-9, which then amplifies the cascade through the activation of downstream caspases-3 and -7 (as reviewed in Ref. 44). These results are consistent with those obtained by etoposide-induced apoptosis in T cells (18). This mechanism is independent of the 20 S proteasome, because preincubation of the cells with lactacystin could not inhibit ionomycin-induced phosphatidylserine externalization. We emphasize that our results do not exclude the involvement of other proteases like caspase-12 and cathepsins L or B in this ionomycin-induced apoptotic pathway (17, 29) downstream of calpain. Upstream there appears to be no reason to implicate additional proteases because AC27P is highly specific for calpains (62),[2] and the lysosomal localization of cathepsins makes them less likely to trigger apoptosis.

The above discussed results can be integrated into the generally accepted model of apoptosis induction (Fig. 8): An increase in the intracellular calcium concentration results in the activation of ubiquitous calpains, which then truncate Bcl-2 family proteins. In particular, activated calpains cleave Bcl-2, resulting in the death-promoting molecule and allowing the translocation of Bax (or itself) to the mitochondria. Bid is also cleaved and presumably also translocated to the mitochondria, amplifying the apoptotic signaling pathway. Targeting of these molecules to mitochondria promotes the liberation of cytochrome *c*, thus activating the intrinsic apoptotic pathway via apoptosome formation and caspase-9 activation. The final outcomes of executioner caspase activation (Fig. 3) are the cleavage of specific substrates such as PARP, DNA degradation (Fig. 2), and finally dismantlement of the cell (Figs. 1 and 2), also manifested in the exposure of phosphatidylserine (Fig. 3). For the first time, a correlation between calpain cleavage of Bcl-2 and Bid in apoptosis of cancer cells has been demonstrated.

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