THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5947–5957, 2004
Printed in U.S.A.
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Calpain-1 Regulates Bax and Subsequent Smac-dependent Caspase-3 Activation in Neutrophil Apoptosis*

Received for publication, August 5, 2003, and in revised form, October 22, 2003
Published, JBC Papers in Press, November 11, 2003, DOI 10.1074/jbc.M308576200

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In the absence and in the resolution of inflammatory responses, neutrophils rapidly undergo spontaneous apoptosis. Here we report about a new apoptosis pathway in these cells that requires calpain-1 activation and is essential for the enzymatic activation of the critical effector caspase-3. Decreased levels of calpastatin, a highly specific intrinsic inhibitor of calpain, resulted in activation of calpain-1, but not calpain-2, in neutrophils undergoing apoptosis, a process that was blocked by a specific calpain-1 inhibitor or by intracellular delivery of a calpastatin peptide. Further support for the importance of the calpastatin-calpain system was obtained by analyzing neutrophils from patients with cystic fibrosis that exhibited delayed apoptosis, associated with markedly increased calpastatin and decreased calpain-1 protein levels compared with neutrophils from control individuals. Additional studies were designed to place calpain-1 into the hierarchy of biochemical events leading to neutrophil apoptosis. Pharmacological calpain inhibition during spontaneous and Fas receptor-induced neutrophil apoptosis prevented cleavage of Bax into an 18-kDa fragment unable to interact with Bcl-x_L. Moreover, calpain blocking prevented the mitochondrial release of cytochrome *c* and Smac, which was indispensable for caspase-3 processing and enzymatic activation, both in the presence and absence of agonistic anti-Fas receptor antibodies. Taken together, calpastatin and calpain-1 represent critical proximal elements in a cascade of pro-apoptotic events leading to Bax, mitochondria, and caspase-3 activation, and their altered expression appears to influence the life span of neutrophils under pathologic conditions.

Neutrophils are important players within the innate immune system. Neutrophil products are often toxic, inasmuch as they are made for killing microorganisms. As targeting of the toxic neutrophil products is not specific, inflammatory responses are associated with more or less tissue damage (1). To reduce neutrophil numbers, for instance after successful removal of the antigenic initiators of an inflammatory response, a safe removal of the cells is required. Such removal occurs through apoptosis, a form of cell death that prevents the release of inflammatory mediators from the dying cell and, therefore, limits tissue damage (2, 3). Clearly, any failure in the process of neutrophil apoptosis would result in additional and/or persistent inflammation. Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases and appears to largely be mediated by overexpression of survival factors for these cells (4, 5). The induction of neutrophil apoptosis during the resolution of a neutrophilic inflammatory response can be mimicked *in vitro* by culturing the cells in the absence of sufficient amounts of survival factors, a process that is called spontaneous apoptosis. Spontaneous neutrophil apoptosis can be enhanced by Fas receptor stimulation (6, 7).

Both spontaneous and Fas receptor-mediated neutrophil apoptosis involves the activation of a family of cysteine proteases, which cut cellular substrates at an obligatory aspartic acid within a preferred sequence (8). Members of this family are the so-called caspases. Caspases have been shown to cut cellular substrates by limited proteolysis that results in either activation or inactivation, but not degradation, of proteins involved in RNA splicing, DNA repair, maintenance of cell structure, and others (9). Their action in apoptosis appears to be critical. Inhibitor studies, however, have provided functional evidence for the importance of other proteases in the execution of apoptosis as well (10). Recently a significant focus has been directed toward calpain, a non-caspase cysteine protease with several isoforms (11, 12).

The ubiquitous calpain isoforms are calpain-1 (calpain I, μ-calpain) and calpain-2 (calpain II, m-calpain), distinguished by their *in vitro* calcium requirements. Both calpain-1 and calpain-2 are heterodimers consisting of a calcium-binding catalytic 80-kDa subunit and a regulatory 30-kDa subunit, which is functionally essential (13). There is accumulating evidence for the involvement of calpain in several physiological processes such as cell-cycle regulation, activation of transcription factors, differentiation, and apoptosis (11, 12).

Calpain has been described as an upstream regulator of apoptosis based on the observation that dexamethasone-induced thymocyte apoptosis was prevented by calpain inhibitors, whereas DNA fragmentation in isolated thymocyte nuclei was not (14). Recently, Bax, a pro-apoptotic member of the Bcl-2 family involved in triggering apoptosis via mitochondria, has been identified as a target of calpain in HL-60 (15) and Jurkat cells (16). Whereas in HL-60 cells Bax cleavage into an 18-kDa fragment occurred several hours after cleavage of poly-(ADP-ribose) polymerase and retinoblastoma protein (15), the results reported in Jurkat cells suggested that the generation of the 18-kDa Bax fragment is an early event required for the induction of apoptosis via mitochondria (16). Because Bax ap-

* This work was supported by Swiss National Science Foundation Grant 31-58916.99 and by the OPO Foundation, Zurich, Switzerland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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pears to be a critical apoptosis-regulating molecule in neutrophils (4, 17, 18), we studied the calpastatin-calpain-system and its importance for Bax cleavage and apoptosis induction in this particular type of granulocytes.

Here, we demonstrate that calpain-1, but not calpain-2, is activated in spontaneous and Fas receptor-mediated apoptosis of neutrophils. We show that calpain-1 activation involves caspase-dependent proteolysis of calpastatin, generates an 18-kDa cleavage product of Bax unable to interact with anti-apoptotic Bcl-x_L, and is required for mitochondrial release of cytochrome *c* and Smac as well as subsequent enzymatic caspase-3 activation and apoptosis. Moreover, neutrophils purified from patients with a systemic inflammatory disease (cystic fibrosis) provide evidence for decreased activity of the calpain-calpastatin system associated with delayed apoptosis.

MATERIALS AND METHODS

Reagents—The caspase inhibitors *N*-benzyloxycarbonyl (z)[1]-Val-Ala-Asp (VAD)-fluoromethylketone (fmk) and z-Ile-Glu-Thr-Asp (IETD)-fmk were purchased from Alexis Corporation (Läufelfingen, Switzerland). Bongkrekic acid, calpastatin-derived and scrambled control peptide, antennapedia-coupled Smac-N7, antennapedia-coupled control peptide (Ant-BH3-A_{78}), as well as purified human calpain-1 and calpain-2 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Isoform-specific inhibitors of calpains were generous gifts from Dr. J. C. Powers (Georgia Institute of Technology, Atlanta, GA) and had the following chemical structures: Z-Leu-Abu-CONH-CH_2-CH(OH)-C_6H_4-3-OC_6H_4(3-CF_3) (compound 28 for calpain-1) and Z-Leu-Abu-CONH-CH_2-2-pyridyl (compound 35 for calpain-2) (19). GM-CSF was purchased from Novartis Pharma GmbH (Nürnberg, Germany). HEK 293T cells overexpressing Smac (20) were obtained from Dr. S. Martin (Trinity College, University of Dublin, Dublin, Ireland). The general calpain inhibitor (L)-3-carboxy-*trans*-2,3-epoxypropionyl-Leu-amido-(4-guanidino)butane ethylester (E64-d) and all other chemicals were, unless stated otherwise, from Sigma (Buchs, Switzerland).

Cells—Peripheral blood neutrophils were purified from healthy normal individuals or patients suffering from cystic fibrosis by Ficoll-Hypaque centrifugation (4, 21). The resulting cell populations contained more than 95% neutrophils. Isolation of eosinophils (22, 23) and generation of T lymphoblasts (24) were performed as previously described. H9, SKW6.4, J16, and CEM cells (25) were grown in RPMI 1640 with 10% heat-inactivated fetal calf serum (complete culture medium). 32D cells were cultured in the presence of IL-3 (26).

Cell Cultures—Cells were cultured at 1×10^6 cells/ml in complete culture medium, and, where indicated, treated with E64-d, calpain-1, or calpain-2 inhibitor (19), calpastatin (27, 28), Smac (29), or control peptides at the indicated concentrations. In other experiments cells were incubated with the caspase-8 inhibitor z-IETD-fmk (50 μM) or the pan-caspase inhibitor z-VAD-fmk (50 μM). Me_2SO was always used as a solvent control. In the experiments in which agonistic anti-Fas receptor monoclonal antibodies (1 μg/ml CH11, Beckman Coulter International S.A., Nyon, Switzerland) were added, the inhibitors were given 1 h before Fas receptor stimulation. The duration of CH11 or GM-CSF (50 ng/ml) stimulation is indicated in each presented experiment.

Determination of Cell Death and Apoptosis—Neutrophil death was assessed by uptake of 1 μM ethidium bromide and flow cytometric analysis (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as previously described (4, 26, 30). To determine whether cell death was apoptosis, redistribution of phosphatidylserine (23, 30), oligonucleosomal DNA fragmentation (4, 23), and morphologic analysis were performed.

In Vitro Autolytic Digestion of Purified Calpain-1—Purified procalpain-1 was dissolved in digestion buffer and incubated at 37 °C for 30 min, according to the instructions from the manufacturer (Calbiochem). The digestions were stopped by adding Tris-glycine loading buffer, and aliquots were subjected to gel electrophoresis and immunoblotting.

Immunoprecipitation—Neutrophils (5×10^7) were washed with PBS and buffer A (50 mM Hepes, pH 7.2, 143 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, phosphatases, and proteases inhibitors), and then lysed in buffer A plus 0.2% Nonidet P-40 on ice for 30 min. Cell lysates were precleared with 100 μl of 50% protein A-Sepharose (Sigma) at 4 °C for 1 h. Immunoprecipitation was performed using 5 μl of affinity-purified rabbit anti-human Bcl-x_L S18 (Santa Cruz Biotechnology, Inc., LabForce, Nunningen, Switzerland) in the presence of 2.5 mg/ml ovalbumin at 4 °C for 2h. Fifty μl of a 50% protein A-Sepharose suspension were added and the immunocomplexes captured on a rotating wheel at 4 °C for 90 min. Immunocomplexes were washed three times in buffer A and boiled at 95 °C for 5 min before loading on 12% NuPAGE gels (Invitrogen, Groningen, Netherlands).

Gel Electrophoresis and Immunoblotting—Neutrophils (5×10^6) were washed with PBS, lysed with radioimmune precipitation assay buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS in PBS) supplemented with a protease inhibitor mixture with frequent vortexing on ice for 40 min, including two freeze/thaw cycles using a methanol/dry ice bath. After a 10-min centrifugation step to remove insoluble particles, equal amounts of the cell lysates were loaded on Tris-glycine gels or NuPAGE gels (Invitrogen). Separated proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The filters were incubated with primary antibodies at room temperature for 2 h or at 4 °C (overnight) in TBS, 0.1% Tween 20, 3% nonfat dry milk. The primary antibodies were polyclonal anti-Bax, detecting amino acids 43–61 (15); polyclonal anti-caspase-3 (both 1/1000 and from BD Biosciences, Pharmingen); monoclonal anti-calpain-1 antibody (1/1000; Chemicon, Temecula, CA); monoclonal anti-calpain-1 antibody (1/200; detecting latent and activated forms (Ref. 31); kind gift of J. S. Elce, Queens University, Kingston, Ontario, Canada); monoclonal anti-calpain-2 antibody (1/500; Sigma); and monoclonal anti-calpastatin antibody (1/400; Chemicon). For loading controls, stripped filters were incubated with a monoclonal anti-β-actin antibody (1/10,000; Sigma). Filters were washed in TBS, 0.1% Tween 20 for 30 min and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Dübendorf, Switzerland) in TBS, 0.1% Tween 20, 5% nonfat dry milk for 1 h. Filters were developed by an ECL technique (ECL Kit, Amersham Biosciences) according to instructions from the manufacturer.

Densitometry Analysis—Cleavage of calpastatin was analyzed by densitometry using nonsaturated chemiluminescence exposed films and NIH Image 1.9 software. OD values of calpastatin bands divided by the OD of the corresponding β-actin band are expressed as percentage of OD(calpastatin)/OD(β-actin) of freshly isolated neutrophils, which was defined as 100%.

Bax/Bcl-x_L in Vitro Binding Studies—One μg of purified plasmids containing sequence verified cDNAs encoding Bax (pCRII) or Bcl-x_L (pBSK$^-$, kind gift of L. Boise) was *in vitro* transcribed and translated in the presence of L-[^{35}S]methionine using a coupled transcription/translation TNT kit (Promega, Catalys AG, Wallisellen, Switzerland) according to instructions from the manufacturer. ^{35}S-Labeled truncated Bax was prepared by *in vitro* digestion of ^{35}S-labeled Bax with purified calpain-1 (Calbiochem) in assay buffer (10 mM HEPES, pH 7.4, 2 mM $CaCl_2$) at 37 °C for 5 h. The reaction was stopped by adding E64-d (50 μM). Potential residual full-length Bax was immunodepleted by adding 10 μl of anti-Bax antisera (N20, Santa Cruz) at 4 °C for 2 h (15), followed by incubation with 25 μl of protein A/G plus-agarose (Santa Cruz). The supernatant was subjected to two further cycles of immunodepletion and finally saved as ^{35}S-labeled truncated Bax. ^{35}S-Labeled Bax intended for co-immunoprecipitation experiments was treated in exactly the same way except that addition of purified calpain-1 and antisera (N20) was omitted. Ten μl of full-length or truncated Bax was incubated with 4 μl of translated Bcl-x_L in 100 μl of buffer (10 mM Tris-HCl, pH 7.4, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Nonidet P-40) at 4 °C for 2 h. One hundred μl of buffer containing 2 μg of polyclonal Bax antisera (BD Biosciences, Pharmingen) was added at 4 °C for 2 h, followed by an additional 2-h incubation with 25 μl of protein A/G plus-agarose (Santa Cruz) at 4 °C. After three washing steps with buffer and two PBS washing steps, the bound proteins were eluted by adding loading buffer and heated at 80 °C for 10 min. An aliquot of the obtained protein solution was loaded on 12% NuPAGE gels (Invitrogen), followed by autoradiography.

Subcellular Fractionation—Freshly purified neutrophils and neutrophils cultured in the presence or absence of E64-d (50 μM) and CH11 antibody for 8 h, were washed with cold PBS and digitonin-permeabilized essentially as described elsewhere (20). Equal volumes of the

[1] The abbreviations used are: z, *N*-benzyloxycarbonyl; fmk, fluoromethylketone; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; IL, interleukin; VDAC, voltage-dependent anion channel; IAP, inhibitor of apoptosis; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GM-CSF, granulocyte/macrophage colony-stimulating factor; HEK, human embryonic kidney; E64-d, (L)-3-carboxy-*trans*-2,3-epoxypropionyl-Leu-amido-(4-guanidino)butane ethylester; r.m.s.d., root mean square displacement; BH, Bcl-2-homology region; TBS, Tris-buffered saline; OD, optical density; MD, molecular dynamics.

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NuPAGE loading buffer-supplemented fractions were loaded on 12% NuPAGE gels and subsequently transferred to polyvinylidene difluoride membranes. Filters were incubated with anti-cytochrome *c* monoclonal antibody (1/500; BD Biosciences, Pharmingen) or anti-Smac polyclonal antisera (1/500; Alexis), and binding was detected by using horseradish peroxidase-conjugated secondary antibodies and ECL. Stripped filters were incubated with anti-voltage-dependent anion channel (VDAC) monoclonal antibody (1/500; Calbiochem).

Enzymatic Caspase Assay—Neutrophils were cultured in the presence or absence of E64-d (50 μM), CH11 antibody, and Smac peptide (50 μM) for 10 h, washed with cold PBS, and subsequently lysed in cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% Chaps, 5 mM dithiothreitol, 0.1 mM EDTA) using a Teflon glass homogenizer (VWR International, Ismaning, Germany) on ice for 10 min. After a 10-min centrifugation step at 10,000 $\times$ *g* at 4 °C, caspase-3-like activity was measured in 10 μl of supernatants as enzymatic conversion of the colorimetric substrate Ac-DEVD-pNA at 405 nm according to the instructions from the manufacturer (QuantiZyme caspase-3 cellular activity assay kit; Biomol, Plymouth Meeting, PA). For controls, we also analyzed caspase-3-like activity of recombinant caspase-3 (Calbiochem) in the presence and absence of the indicated concentrations of E64-d and 50 μM Ac-DEVD.

Confocal Laser Scanning Microscopy—Cytospins were made from freshly purified neutrophils and neutrophils cultured in the presence or absence of E64-d (50 μM) and CH11 antibody for 12 h. Cells were fixed in 4% paraformaldehyde at room temperature for 15 min and washed four times in PBS, pH 7.4. Permeabilization of cells was performed with 0.05% saponin in blocking buffer (3% bovine serum albumin in PBS) at room temperature for 5 min and with acetone at −20 °C for 15 min. To prevent nonspecific binding, slides were incubated in blocking buffer at room temperature for 1 h. Indirect immunostainings for cytochrome *c*, Smac, and VDAC were performed at room temperature for 1 h by using the following primary antibodies: anti-cytochrome *c* monoclonal antibody (1/100; diluted in blocking buffer), anti-Smac polyclonal antisera (1/100), and anti-VDAC monoclonal antibody (1/200). Incubation with appropriate TRITC- and FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Milan Analytica, La Roche, Switzerland) was performed in the dark at room temperature for 1 h. The anti-fading agent Slowfade (Molecular Probes) was added, and the cells were covered by coverslips. The slides were analyzed by confocal laser scanning microscopy (LSM 510, Carl Zeiss, Heidelberg, Germany) equipped with argon and helium-neon lasers.

Molecular Protein Modeling—The models were based on the NMR structure of Bax (Protein Data Bank code 1F16) (32). The NH_2-deleted Bax was built by simply removing the first 32 amino acids from the three-dimensional structure of the conformer number 1. All calculations were carried out with the AMBER 6 program (33). The all-atom AMBER force field (34) and the TIP3P model (35) were used for the proteins and the water molecules, respectively. The dielectric constant was set equal to 1. Long range electrostatic interactions were estimated by means of the particle mesh Ewald method (36), whereas the short range electrostatic and van der Waals interactions were truncated within a 12-Å cut-off. Constant temperature pressure was achieved by coupling the system with a Berendsen thermostat (T = 300 K; coupling constant = 1.0 ps) and barostat (P = 1 atm; coupling constant = 1.0 ps) (37). Full-length and truncated Bax were immersed in a 69 $\times$ 58 $\times$ 51-$Å^3$ box containing ~6000 water molecules, to account for the effects of the explicit solvent. The positions of the water molecules were equilibrated by running 5000 geometry optimization steps and 30-ps molecular dynamics (MD) simulations. The two systems were further studied by carrying out 2-ns MD simulations. Because a stable conformation was not achieved even after 2 ns of MD simulations, the NH_2-deleted model was simulated up to 3.2 ns.

RESULTS

Spontaneous and Fas Receptor-mediated Neutrophil Apoptosis Require Calpain-1 but Not Calpain-2—To determine whether calpain has a functional role within apoptotic pathways in neutrophils, we used the previously characterized pan-calpain inhibitor E64-d (14, 38) in an *in vitro* system of neutrophil apoptosis. As shown in Fig. 1*A*, E64-d delayed spontaneous and Fas receptor (CH11)-mediated neutrophil death in a dose-dependent manner. The data represent cell death numbers of 24-h cultures, but the effect of E64-d was also observed in 12-, 36-, and 48-h cultures (data not shown). E64-d also inhibited phosphatidylserine redistribution (Fig. 1*B*), DNA fragmentation (Fig. 1*C*), and morphological changes associated with apoptosis (Fig. 1*D*), suggesting that the type of neutrophil death blocked by E64-d was apoptosis. Several lysosomal (ammonium chloride, chloroquine) and cathepsin inhibitors (compound 28 (Ref. 19)) had no effect in this system (data not shown). To confirm the potential role of calpain in the regulation of neutrophil apoptosis, we applied cell-permeable inhibitors that block specific calpain isoforms. Whereas a spe-

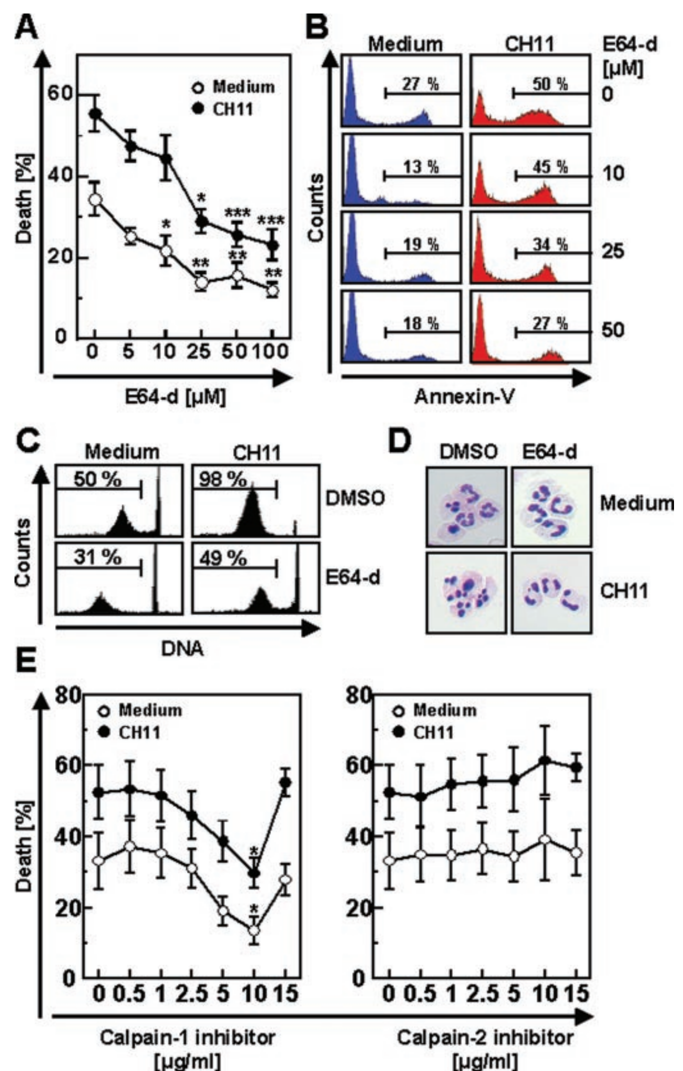

FIG. 1. **Inhibition of calpain-1 blocks spontaneous and Fas receptor-mediated apoptosis of neutrophils.** *A*, death assay in the presence or absence of Fas receptor activation (CH11); a dose-dependent reduction of cell death by E64-d was seen in 24-h neutrophil cultures. Values are means ± S.E. of seven independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *B*, phosphatidylserine redistribution assay in the presence or absence of Fas receptor activation (CH11); a dose-dependent reduction of annexin V binding by E64-d was seen in 5-h neutrophil cultures (propidium iodide negative subpopulation). The results are representative of five independent experiments. *C*, DNA fragmentation assay in the presence or absence of Fas receptor activation (CH11); E64-d (50 μM) inhibited the occurrence of hypoploid DNA in 10-h neutrophil cultures under both conditions. The results are representative of five independent experiments. *D*, neutrophil morphologic analysis in the presence or absence of Fas receptor activation (CH11); E64-d (50 μM) reduced the numbers of neutrophils with an apoptotic phenotype (reduced cell volume and nuclear condensation) in 10-h cultures under both conditions. Cells were stained with Giemsa-May-Grünwald (Diff-Quik) (original magnification, ×1000). The results are representative of five independent experiments. *E*, death assay in the presence or absence of Fas receptor activation (CH11); a dose-dependent reduction of cell death by specific calpain-1 but not calpain-2 inhibitor was seen in 24-h neutrophil cultures. Values are means ± S.E. of five independent experiments (*, $p < 0.05$).

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cific calpain-1 inhibitor (19) dose-dependently delayed neutrophil death, we observed no effect when we used a specific calpain-2 inhibitor (19) in the same system, even at concentrations that exceeded the K_i more than 500 times (Fig. 1*E*). Moreover, because active calpains result as a consequence of autolytic digestion of their respective proforms (11), we also performed immunoblotting and obtained evidence for calpain-1 but not calpain-2 activation in cultured untreated and CH11-activated neutrophils (data not shown). Taken together, these data suggest that calpain-1 but not calpain-2 is involved in the regulation of neutrophil apoptosis.

Critical Role for Calpastatin in Regulating Calpain-1 Activity and Neutrophil Apoptosis—Calpastatin is a highly specific endogenous inhibitor of calpain and to date has not been demonstrated to inhibit the activity of any other protease family (27, 28). As demonstrated in Fig. 2*A*, freshly purified neutrophils expressed several isoforms of calpastatin. In agreement with previously published work using other cellular systems (39, 40), the molecular sizes of these isoforms were 110, 108, 90, 75, and 41 kDa. The most abundant isoform expressed in neutrophils is 41 kDa in size. Culturing of the cells resulted in reduced levels of all isoforms, suggesting proteolysis of the protein during spontaneous apoptosis. Treatment with anti-Fas antibody appeared to accelerate this process, because only traces of the 41-kDa isoform were detectable in neutrophils that had been cultured for 15 h (Fig. 2*A*).

To demonstrate that calpain-1 activation is directly regulated by calpastatin, we incubated freshly isolated neutrophils with a specific inhibitory peptide, containing 27 amino acids that represent the minimal inhibitory motif of calpastatin (27, 28), or with a scrambled control peptide. Because calpain-1 appeared to be part of an apoptotic pathway in neutrophils, we hypothesized that conservation of calpastatin activity by applying the calpastatin peptide should delay apoptosis in these cells. Indeed, as shown in Fig. 2*B*, calpastatin but not scrambled control peptide delayed spontaneous and Fas receptor-mediated neutrophil death, demonstrating that calpastatin directly controls calpain-1 activity. Moreover, the data suggest that the cytosolic free calcium levels present in neutrophils (~120 nM (Ref. 41)) are sufficient for activation of calpain-1. Stimulation of the Fas receptor by agonistic antibodies was not associated with rapid increases in cytosolic free calcium levels (data not shown), further indicating that calpain-1 activation does not require additional calcium.

Although proteolytic activity of caspases appears to be required, direct cleavage of procalpain-1 into its active form occurs via autoactivation (31). Thus, it is unlikely that procalpain-1 is a direct target for caspases. We therefore investigated whether caspases might regulate levels of calpastatin. If levels of calpastatin were critical within a regulatory mechanism for controlling calpain-1 activity, calpastatin levels should rapidly change upon a death stimulus. Indeed, as shown in Fig. 2*C*, Fas receptor activation markedly reduced calpastatin levels within 4 h, a process that was blocked by z-IETD-fmk and the pan-caspase inhibitor z-VAD-fmk, which also preferentially inhibits caspase-8 (42). In the absence of death receptor stimulation, calpastatin levels decreased by ~25% and both caspase inhibitors blocked this process. Interestingly, calpastatin levels even accumulated in the presence of z-VAD-fmk. Together, the data suggest that reduction of calpastatin levels is an early and regulatory event upon apoptosis induction that most likely involves caspase-8 and is not the consequence of increased overall proteolytic activity during later stages of apoptosis.

The Calpastatin-Calpain System in Apoptotic Pathways Is Cell Type-restricted and Dysregulated in Neutrophils under Inflammatory Conditions—We next investigated whether the

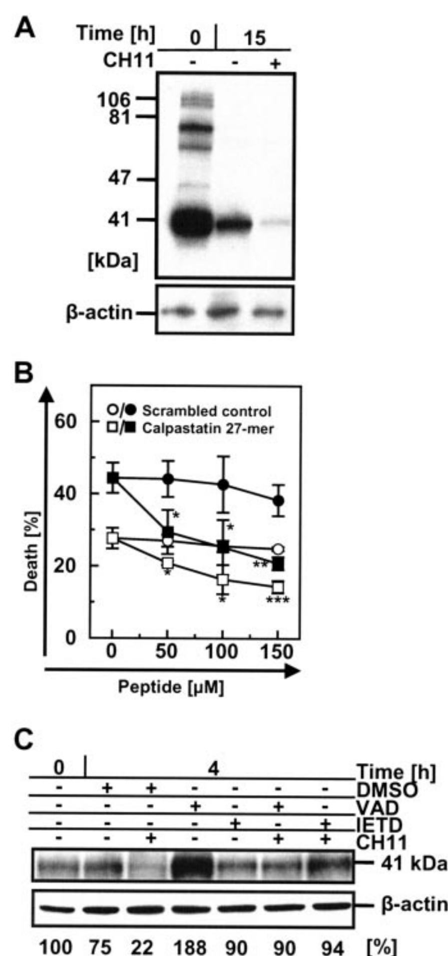

FIG. 2. **Caspase-dependent proteolysis of calpastatin in early spontaneous and Fas receptor-mediated neutrophil apoptosis.** *A*, several isoforms of calpastatin were present in freshly isolated neutrophils. Apoptosis was associated with a dramatic reduction of expression that involves all isoforms. *B*, death assay ($n = 3$) in the presence (*closed symbols*) or absence (*open symbols*) of Fas receptor activation (CH11) in 24-h neutrophil cultures (means ± S.E.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$): A dose-dependent reduction of cell death by the calpastatin peptide, but not with the scrambled control, was seen. *C*, Fas receptor activation resulted in lowering of the calpastatin levels within 4 h. In contrast, z-VAD-fmk (50 μM) treatment increased calpastatin levels. β-Actin protein expression demonstrates equal loading and results of the densitometry analysis of the calpastatin levels in percent is presented *below* the immunoblots. The results are representative of at least three independent experiments.

calpastatin-calpain system plays a general role in the apoptosis regulation of growth factor-dependent and/or Fas-sensitive cells. In 24-h eosinophil cultures, E64-d completely blocked Fas receptor-mediated death. In contrast, neither Fas receptor-mediated death nor death induced by cytokine withdrawal was blocked by E64-d in IL-2-dependent T lymphoblasts or IL-3-dependent mouse 32D cells (data not shown). Moreover, we tested the capacity of E64-d to block Fas receptor-mediated death in several Fas-sensitive so-called type I and type II cells (H9, SKW6.4, J16, and CEM) (25). In all these cells, we observed no effect of E64-d on Fas receptor death (data not shown). This suggests that the role of calpain as a key molecule within apoptotic pathways initiated by cytokine withdrawal or Fas receptor activation is cell type-restricted.

Cystic fibrosis is a disease in which patients suffer from bacterial infections of the lung. In agreement with previously published work (4), we observed delayed spontaneous cell death of purified blood neutrophils *ex vivo* (Fig. 3*A*). When we compared the levels of expression of calpastatin and procal-

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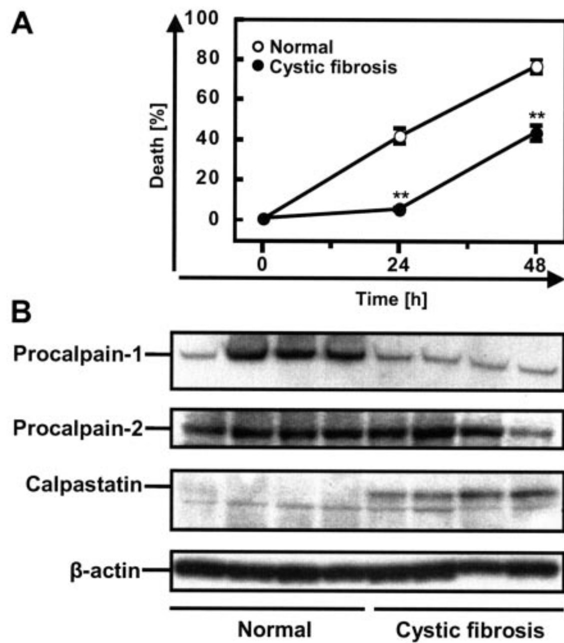

FIG. 3. **Delayed neutrophil death in cystic fibrosis is associated with reduced calpain-1 and increased calpastatin levels.** *A*, death assay of cultured blood neutrophils derived from cystic fibrosis patients ($n = 7$) and from normal control individuals ($n = 7$). Values are means ± S.E. (**, $p < 0.01$). *B*, the levels of procalpain-1 in neutrophils were decreased in the group of cystic fibrosis patients compared with the majority of normal control individuals. In contrast, levels of procalpain-2 were the same in the two groups. In addition, high calpastatin expression (41-kDa isoform) was associated with cystic fibrosis. Equal loading of the gel was ensured by detection of β-actin on the stripped filter.

pain-1 protein in neutrophils derived from cystic fibrosis patients with those from normal control individuals, we observed striking differences. Whereas calpain-2 levels appeared to be similar, cystic fibrosis neutrophils contained markedly increased levels of calpastatin and significant decreased amounts of the 80-kDa proform of calpain-1 compared with normal neutrophils (Fig. 3*B*). Although the mechanisms responsible for these findings remain to be investigated, it is likely that the differences in the expression of calpastatin and calpain contribute to the observed delay of neutrophil apoptosis in cystic fibrosis, and, perhaps, in other bacterial infectious diseases. Moreover, the dysregulated expression of calpastatin and calpain in neutrophils, which exhibit delayed apoptosis, further suggests a key role for these two proteins in the regulation of neutrophil apoptosis.

The Proteolytic Activity of Calpain-1 Involves Cleavage of Bax—Bax is a pro-apoptotic member of the Bcl-2 family that is highly expressed in neutrophils (4, 17, 18) and appears to be critical for the breakdown of the mitochondrial potential (43–46). Moreover, Bax cleavage by calpain was reported in drug-induced apoptosis (cleavage sequence: FIQD, at amino acids 30–33) (15). As demonstrated in Fig. 4*A*, freshly isolated neutrophils contained the full-length 21-kDa form of Bax. Spontaneous neutrophil apoptosis (12-h cultures) was associated with Bax cleavage into an 18-kDa fragment, a process that was accelerated in the presence of agonistic anti-Fas receptor antibodies. Cleavage of Bax is detectable in untreated neutrophil cultures after 6 h (data not shown). The generation of the 18-kDa Bax fragment was at least partially blocked by E64-d in cultured neutrophils both in the presence and absence of anti-Fas receptor antibodies (Fig. 4*A*). Consistent with the ability of caspase inhibitors to block calpain-1 activation, Bax cleavage was less efficient in neutrophils pretreated with z-VAD-fmk and z-IETD-fmk, regardless of whether the Fas receptor was activated or not (Fig. 4*B*).

Bcl-x_L Is Able to Physically Interact with Full-length Bax but Not with Its 18-kDa Fragment—To understand the molecular alterations of the Bax molecule induced by calpain-1-mediated cleavage, we performed dynamic molecular modeling of the 18-kDa Bax fragment. The model based on the NMR-structure of full-length human monomeric Bax (32). In Fig. 5*A*, the root mean square displacements (r.m.s.d.) of the backbone atoms were plotted *versus* the simulation time (upper panels). Full-length Bax appeared to achieve a stable conformation after ~700 ps of MD simulations (*left upper panel*; r.m.s.d. ≈ 3.2 Å with respect to the minimized NMR structure). In contrast, the NH_2-terminal truncated 18-kDa Bax did not achieve a stable conformer until 2.5 ns of MD simulations (*right upper panel*). Longer MD simulations (up to 3.2 ns) were also not sufficient (data not shown).

As demonstrated in the *lower two panels* of Fig. 5*A*, the main contribution to the overall r.m.s.d. was the result of the NH_2 terminus domain. In the 21-kDa Bax molecule, only some amino acids (residues 36–55) showed a r.m.s.d. greater than 3 Å in the 21-kDa protein (*left lower panel*), explaining the high thermal protein stability over 2 ns of MD simulations. Compared with full-length Bax, the 18-kDa Bax fragment had a higher average r.m.s.d. in the NH_2 terminus domain (*right lower panel*). Moreover, there were more domains showing an average r.m.s.d. higher than 3 Å (amino acids 51–61, 129–132, and 169–172). One of these amino acid stretches largely represents the BH3 domain (residues 53–63), which in turn is a part of α-helix 2 (residues 53–72). This is of interest, because the BH3 domain has been demonstrated to interact with anti-apoptotic members of the Bcl-2 family (32). Fig. 5*B* shows a superimposition of the backbone structures of full-length and truncated Bax. The magnification (*right panel*) demonstrates the calculated changed conformation of the BH3 domain and the amino acid residues important for the interaction with anti-apoptotic Bcl-2 family members (32). The most obvious change in orientation involved the two leucines within the α-helix 2 (amino acid positions 59 and 63) that appeared to be directed toward the hydrophobic core of the protein upon cleavage by calpain-1.

Based on our results obtained by dynamic molecular modeling, we hypothesized that, in contrast to full-length Bax, the 18-kDa Bax fragment cannot physically interact with anti-apoptotic members of the Bcl-2 family. Neutrophils have been described to express Bcl-x_L (4, 17) but not Bcl-2 (4, 6). To test the hypothesis, Bcl-x_L and Bax were *in vitro* transcribed and translated in the presence of [^{35}S]methionine, leading to the generation of labeled proteins. Truncated Bax was generated by *in vitro* digestion of ^{35}S-labeled Bax with recombinant calpain-1. Bcl-x_L was incubated with Bax or truncated Bax. Fig. 5*C* demonstrates the results obtained by autoradiography following separation of anti-Bax immunocomplexes by SDS-page. Co-immunoprecipitation of Bcl-x_L was seen with full-length Bax. In contrast, truncated Bax was unable to associate with Bcl-x_L. The same system was used to exclude the possibility that z-VAD-fmk inhibits calpain-1, which did not block the formation of truncated Bax (data not shown). Therefore, the inhibition of calpastatin cleavage by z-VAD-fmk (Fig. 2*C*) was unlikely as a result of inhibition of calpain-1.

We also performed co-immunoprecipitation experiments in intact neutrophils. Bcl-x_L was immunoprecipitated, and the presence of Bax molecules was investigated by subsequent immunoblotting using anti-Bax antibody. As shown in Fig. 5*D*, both full-length and truncated Bax were readily detectable in neutrophils following a 24-h culture period before immunopre-

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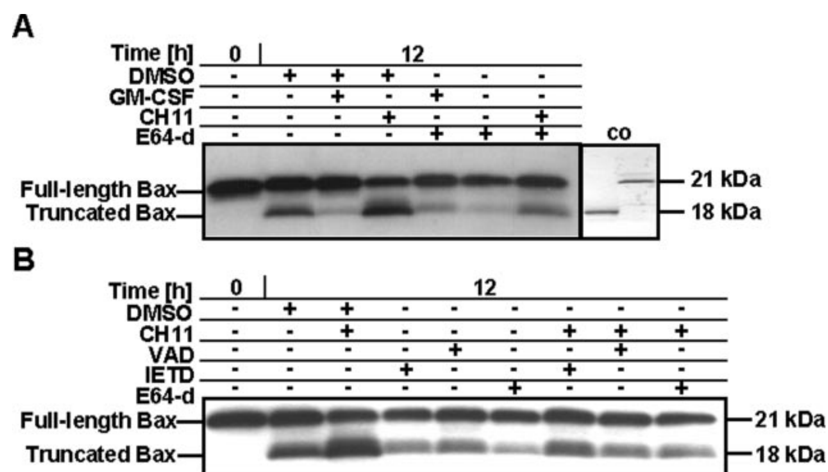
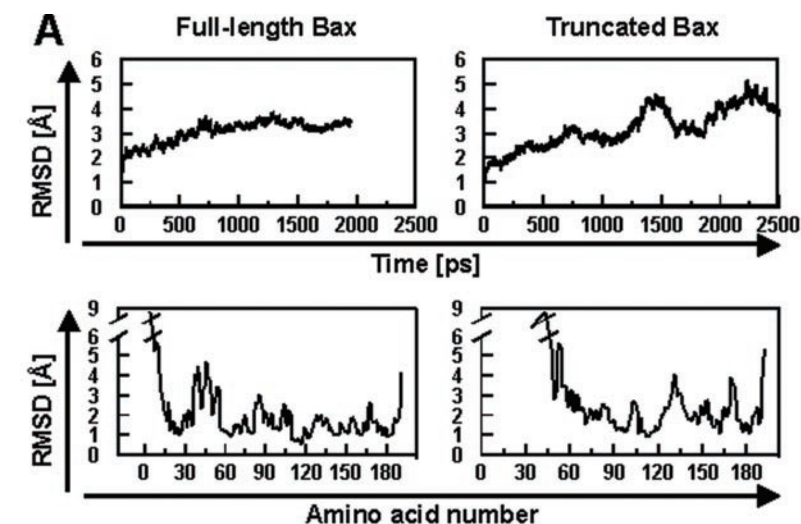
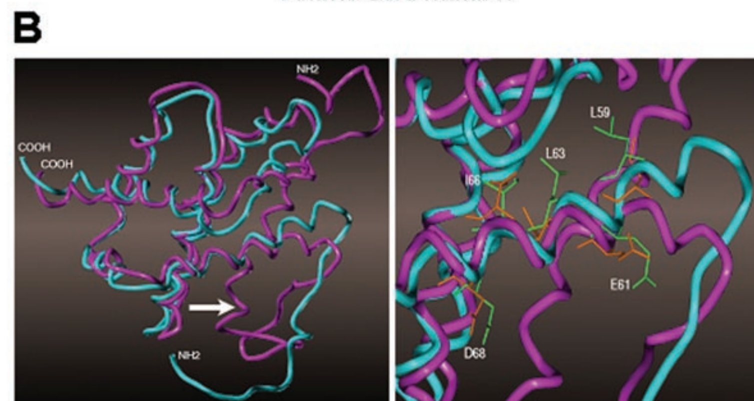


FIG. 4. **Calpain cleaves Bax into an 18-kDa fragment in spontaneous and Fas receptor-mediated neutrophil apoptosis.** *A*, Bax cleavage was enhanced upon Fas receptor activation (CH11) and reduced in the presence of E64-d (50 μM) or GM-CSF (50 ng/ml). The 18-kDa truncated form of Bax was generated by *in vitro* digestion of ^{35}S-labeled full-length Bax with purified calpain-1 and served as a control (*co*). *B*, complete (z-VAD-fmk, 50 μM) and partial (z-IETD-fmk, 50 μM) block of Bax cleavage in the presence of caspase inhibitors was observed, regardless of whether the Fas receptor was activated or not. Results are representative of at least three independent experiments.





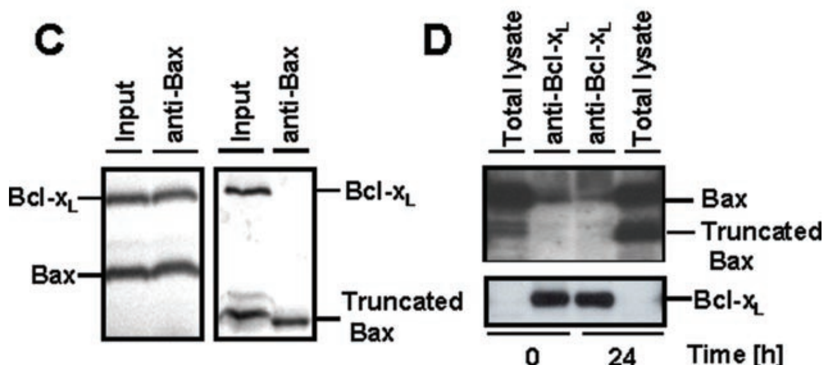


FIG. 5. **A conformational change of the BH3 domain upon calpain-mediated Bax cleavage results in the inability of truncated Bax to interact with Bcl-x_L.** *A*, r.m.s.d. of full-length Bax (*left upper panel*) and truncated Bax (*right upper panel*) backbone atoms. The NH_2-deleted form of Bax did not reach a stable conformer even after 2.5 ns of MD simulations. The model fluctuated around 3 and 4 Å and indicated that truncated Bax might require a lipophilic (membrane) environment to achieve a stable conformation. The *lower panels* show the average r.m.s.d. of each amino acid backbone. Of particular interest is the fact that the α-helix 2 (part of the BH3 domain) gained higher flexibility in truncated Bax (amino acids 53–72). *B*, superimposition of the backbone structures of full-length Bax (in *magenta*) and truncated Bax (in *blue*) with the calpain cleavage site highlighted by the *arrow* (*left panel*). Higher magnification of the BH3 domain (*right panel*) demonstrates the markedly changed conformation and the amino acid residues important for the interaction with Bcl-x_L (note, in particular, the two leucines at positions 59 and 63). Amino acid residues of full-length Bax are given in *brown*, residues of truncated Bax in *green*. *C*, immunoprecipitation of ^{35}S-labeled proteins. Full-length Bax but not truncated Bax physically interacted with Bcl-x_L. Identical results were obtained in three additional experiments. *D*, full-length but not truncated Bax was present in Bcl-x_L immunoprecipitates of 24-h cultured neutrophils (*upper panel*, *lane 3*). The total neutrophil lysate (*lane 4*) demonstrates that the cells contained both full-length and truncated Bax. The *lower panel* shows that, although Bcl-x_L is difficult to detect in neutrophils by regular immunoblotting techniques, it can efficiently be enriched by immunoprecipitation. Identical results were obtained in two additional independent experiments. Moreover, the lack of association between truncated Bax and Bcl-x_L was also demonstrated in staurosporine-treated HL-60 cells (data not shown).

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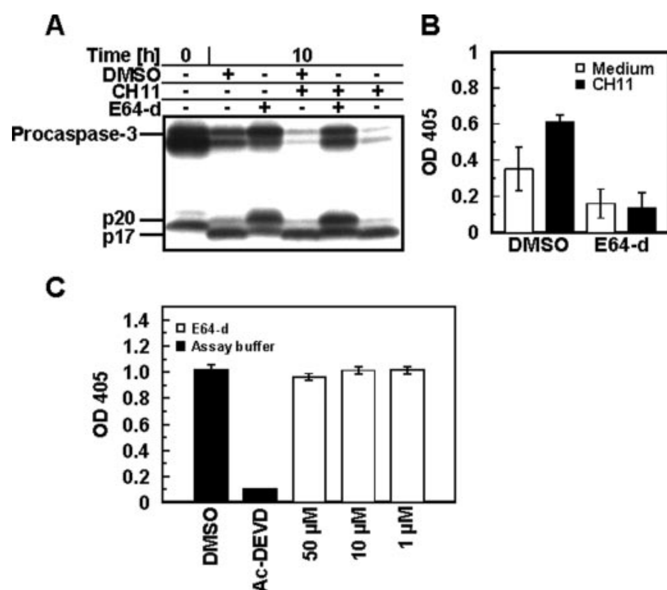

FIG. 6. **Pharmacological inhibition of calpain blocks caspase-3 processing and activation.** *A*, apoptosis was associated with the occurrence of a 17-kDa fragment. E64-d (50 μM) treatment resulted in the accumulation of a 20-kDa fragment, regardless of whether the Fas receptor was activated or not. Results are representative of three independent experiments. *B*, caspase-3 activity assay; increases in the enzymatic activity were detectable in neutrophils undergoing spontaneous apoptosis, a process that was accelerated by Fas receptor activation. Inhibition of calpains by E64-d (50 μM) blocked the activation of caspase-3, regardless of whether the Fas receptor was activated or not. Values are means ± S.E. of five independent experiments. *C*, activity of recombinant caspase-3 was not affected by the indicated concentrations of E64-d. In contrast, Ac-DEVD (50 μM) completely blocked the enzymatic activity of recombinant caspase-3. Values are means ± S.E. of three independent experiments.

cipitation (*upper panel*, *lane 4*). In contrast, Bcl-x_L was hardly seen under these conditions, but was clearly enriched following immunoprecipitation (*lower panel*, *lanes 2* and *3*). Full-length but not truncated Bax was present within the Bcl-x_L immunoprecipitates (*upper panel*), again suggesting that truncated Bax is unable to associate with Bcl-x_L. Identical results were obtained using staurosporine-treated HL-60 cells (data not shown). These biochemical data are in agreement with the results obtained by dynamic molecular modeling and suggest that truncated Bax does not interact with Bcl-x_L as a result of a conformational change in the α-helix 2 of Bax, which contains the BH3 domain, upon dissection of α-helix 1 by calpain-1.

Critical Role for Calpain-1-dependent Mitochondrial Release of Smac in Caspase-3 Processing and Activation—Caspase-3 is a critical effector caspase in neutrophil apoptosis (6, 17, 18). As shown in Fig. 6*A*, in freshly purified neutrophils, a 19-kDa cleavage product of procaspase-3 was seen. The active 17-kDa fragment (47, 48), however, was not detectable. Culturing the cells for 10 h resulted in the appearance of the 17-kDa form, and cleavage of caspase-3 was accelerated in anti-Fas receptor antibody-treated neutrophils. In contrast, the unfragmented procaspase-3 (double band at 33 and 31 kDa) was conserved in E64-d-treated cells, suggesting that blocking of calpain-1 inhibited caspase-3 processing. Interestingly, a 20-kDa fragment and an additional 18-kDa fragment accumulated in cells in which calpain-1 had been inhibited. However, no formation of the 17-kDa form of caspase-3 was observed in E64-d-treated cells. The 20-kDa fragment might be the result of direct cleavage of caspase-3 by caspase-8 without contribution of the mitochondrial pathway (49). Furthermore, caspase-3-like DEVDase activity was strongly suppressed in neutrophils in the presence of E64-d, regardless of whether the Fas receptor was activated or not (Fig. 6*B*). Thus, drug-induced calpain inhibition and the subsequent apoptosis block appeared to correlate with inhibition of processing of both procaspase-3 and its 20-kDa fragment, resulting in no or only little enzymatic activity.

Together with the Bax data, we hypothesized at this point that calpain is required for caspase-3 activation and subsequent apoptosis via a mitochondria-derived mediator. Smac is a recently identified pro-apoptotic factor released from mitochondria into the cytosol upon apoptosis induction (50, 51). Separation of cytosol and membranes and subsequent detection of Smac protein demonstrated its presence and redistribution during both spontaneous and Fas receptor-mediated apoptosis in neutrophils (Fig. 7*A*). The VDAC (43, 44) was used as a mitochondrial marker. In addition to Smac, cytochrome *c* was also released from mitochondria into the cytosol of cultured neutrophils and activation of the Fas receptor enhanced this process. Pharmacological inhibition of calpain-1 by E64-d blocked both Smac and cytochrome *c* release into the cytosol (Fig. 7*A*). These data were confirmed by using fluorescence immunostaining and microscopic analysis. As shown in Fig. 7*B*, Smac and cytochrome *c* co-localized to mitochondria and a punctate pattern was observed in freshly isolated neutrophils. However, cell culturing for 12 h revealed a diffuse cytosolic staining pattern of the two proteins, indicating release of Smac and cytochrome *c* from mitochondria, in ~50% of the neutrophils. Following Fas receptor activation, ~90% of the neutrophils demonstrated evidence for Smac and cytochrome *c* release. When E64-d was added, the punctate mitochondrial staining pattern of Smac and cytochrome *c* was preserved, even in neutrophils activated via the Fas receptor. Taken together, the results presented in Fig. 7 (*A* and *B*) suggest that calpain inhibition does not allow the release of the two pro-apoptotic factors Smac and cytochrome *c* from mitochondria.

To gain insights into the functional relevance of Smac release for the execution of apoptosis in neutrophils, we incubated the cells with an antennapedia-coupled peptide containing the NH_2-terminal "AVPI" motif of Smac. It has previously been reported that this sequence represents the minimal motif of Smac that is able to bind to the baculoviral domain of inhibitors of apoptosis (IAPs) and consequently to counteract IAP inhibition on caspases (29). As shown in Fig. 7*C*, the Smac but not an antennapedia-coupled control peptide accelerated spontaneous neutrophil death in a dose-dependent manner. Addition of E64-d had no effect in this system, further supporting the data reported in this article that suggest that calpain controls apoptotic pathways proximal to mitochondria activation in neutrophils. Moreover, addition of the Smac peptide accelerated caspase-3 activation in spontaneous and Fas receptor-mediated apoptosis and also allowed processing of the accumulating inactive 20-kDa fragment in the presence of E64-d as indicated by increased amounts of the 17-kDa fragment (Fig. 7*D*). These findings were supported by measuring the caspase-3-like DEVDase activity, which increased after addition of the Smac peptide even in the presence of E64-d (data not shown).

DISCUSSION

In this study, we investigated calpain functions in apoptotic pathways of neutrophils and report the following new findings. 1) Blocking of calpain-1 activity by highly specific inhibitors delays spontaneous and Fas receptor-mediated apoptosis. 2) Calpain-1 activation correlates with decreased calpastatin levels, which are regulated in a caspase-8-dependent manner. 3) Calpain-1 cleaves Bax resulting in a cleavage product unable to bind Bcl-x_L, most likely as a result of conformational changes within the BH3 domain. 4) Calpain-1 mediates cytochrome *c* and Smac release from mitochondria into the cytosol. 5) Enzymatic activity of caspase-3 requires calpain-1, possibly by

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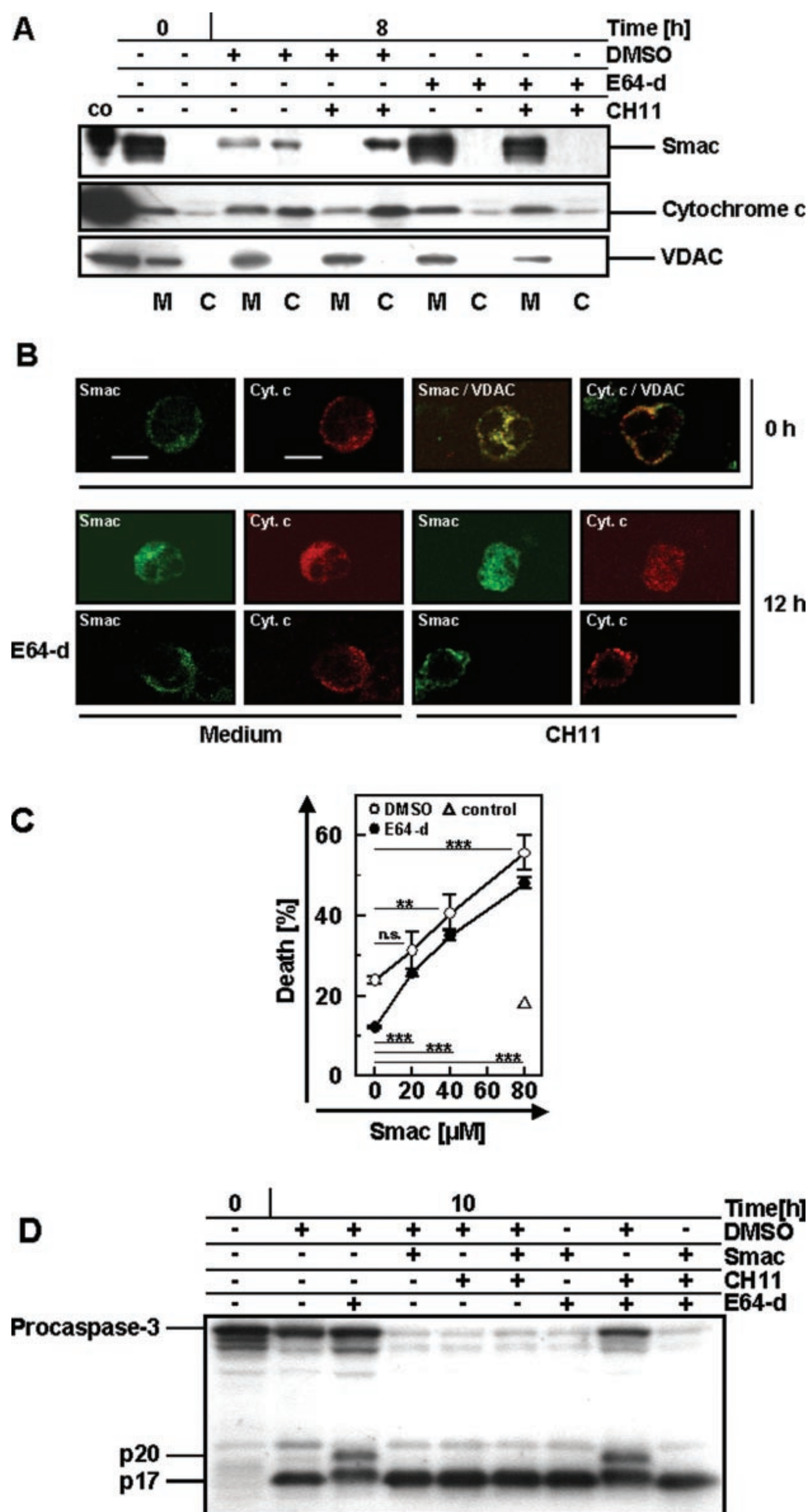

FIG. 7. **Calpain is required for the mitochondrial release of Smac, which mediates caspase-3 processing and activation.** *A*, cytosolic (*C*) and mitochondrial (*M*) fractions were investigated by immunoblotting. Release of mature Smac and cytochrome *c* from the mitochondria into the cytosol upon spontaneous and Fas receptor-induced apoptosis were blocked by E64-d (50 μM). As controls (*co*), HEK 293T whole-cell lysate overexpressing Smac and purified cytochrome *c* were applied. The stripped filter was used for anti-VDAC immunoblotting to confirm the absence of mitochondrial proteins in the cytosolic fraction. Identical results were obtained in two additional experiments. *B*, confocal microscopy: Neutrophils were cultured under the same conditions as seen in *A* for 12 h. Smac and cytochrome *c* were localized with VDAC, demonstrating that they are mitochondrial proteins in freshly isolated neutrophils. E64-d preserved the punctate pattern in cultured neutrophils under both CH11-treated and -untreated conditions, whereas we observed a diffuse staining in the absence of calpain inhibition. The *bar* represents 10 μm. No detectable staining was observed by using isotype-matched control antibodies (data not shown). Results are representative of five independent experiments. *C*, death assay in the presence and absence of E64-d (50 μM) in 24-h neutrophil cultures. Smac dose-dependently induced cell death under both conditions. A antennapedia-coupled non-functional peptide served as control. Values are means ± S.E. of four independent experiments (**, $p < 0.01$; ***, $p < 0.001$). *D*, Smac overcame the block of caspase-3 processing and activation caused by pharmacological inhibition of calpain. The accumulating 20-kDa fragment of caspase-3 in E64-d-treated (50 μM) neutrophils was not seen as soon as Smac peptide (25 μM) was added. Instead the 17-kDa form appeared. Results are representative of three independent experiments.

Smac-mediated inactivation of IAPs. 6) Decreased calpain-1 and increased calpastatin expression levels are associated with delayed neutrophil apoptosis in a bacterial infectious disease, suggesting that the reported calpain-1-mediated pro-apoptotic pathway is important *in vivo* and dysregulated under pathologic conditions.

The physiologic role of calpain is poorly understood, but its presence and conservation in almost all mammalian cells make it reasonable to assume that this cysteine protease is an important enzyme (11, 12). Indeed, deletion of the gene for the small subunit of calpain in mice is embryonic lethal, suggesting that calpain is essential for life (13). The involvement of the calpain-calpastatin system in apoptosis was suggested by numerous studies involving different cellular systems, including immature T (14) and B cells (52, 53). A role in mature T cell apoptosis of patients with HIV infection (54) and in spontaneous neutrophil apoptosis (10) has also been suggested based on inhibitor studies, but its exact role remained unclear because of its unknown substrate(s) in apoptosis pathways. In particular, there have been contrasting data whether calpain and caspases work in a cooperative manner (10, 16, 55) or whether they independently act in different apoptotic pathways (56).

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In the Fas receptor-mediated death pathway, it has not been understood whether calpain is directly or indirectly (by prevention of Fas ligand expression (Ref. 54)) involved in the apoptotic pathway(s). Recent findings observed in HL-60 (15) and Jurkat (16) cells point to the possibility that Bax is a target of calpain, although it remained to be investigated whether this is an early regulatory (16) or late (10, 15) event in apoptosis. Moreover, because the mitochondrial involvement in the Fas pathway has been questioned (57, 58), it was unclear whether Fas receptor-mediated apoptosis would indeed require Bax cleavage. Here, we demonstrate that the Fas pathway requires calpain and cleavage of Bax in neutrophils (and most likely in eosinophils), but not in other cell types including other growth factor-dependent cells (human T-lymphoblasts and mouse 32D cells) or previously described type II cells (25). Although it is clear that the Fas pathway described in this paper is cell type-dependent, it remains to be investigated whether non-granulocytic cells such as immature T (14) and B (52, 53) cells also use at least some components of it.

Cleavage of Bax by calpains has been reported to generate a NH_2-terminal truncated molecule of 18 kDa with enhanced pro-apoptotic potential (16). We monitored Bax expression with an antibody detecting full-length Bax and its 18-kDa fragment. Cleavage of Bax was not detectable in non-apoptotic cells and correlated in neutrophil cultures with the numbers of apoptotic cells. Consequently, Fas receptor activation resulted in increased Bax cleavage compared with untreated neutrophils at a given time, and this was blocked by pharmacological inhibition of calpain-1. These data suggested that both spontaneous and Fas receptor-mediated neutrophil apoptosis require the calpain-Bax pathway and are in agreement with previously published work suggesting that Bax is a key regulator of neutrophil apoptosis (4, 17, 18). In addition to decreased Bax levels (4, 17), we observed increased calpastatin and decreased calpain-1 expression levels in cystic fibrosis neutrophils, implying that diminished Bax activation caused by reduced calpain-mediated cleavage may also contribute to delayed neutrophil apoptosis and subsequent accumulation of these effector cells under inflammatory conditions. Therefore, we investigated the functional consequences of Bax cleavage in spontaneous and Fas receptor-mediated neutrophil apoptosis in more detail.

To understand how the cleaved form of Bax might enhance its pro-apoptotic activity (16), we compared the full-length and the 18-kDa form of Bax by dynamic molecular modeling based on the three-dimensional structure of the full-length molecule, in which α-helices 1, 4, 5, and 6 create a hydrophobic core and the hydrophobic residues of the BH3 domain (α-helix 2) are directed toward it (32). The hydrophobic core in this conformation appears to be required for formation of heteromeric complexes of Bax with anti-apoptotic members of the Bcl-2 family, such as Bcl-x_L and Bcl-2 via the BH3 domain (32). The calpain-cleavage site (15) is located at the COOH-terminal end of α-helix 1, thus the NH_2-terminal α-helix 1 is not present in truncated Bax. The superimposition of the two proteins revealed that Bax does not change much in its main structure, with the exception of the NH_2-terminal part of α-helix 2, encompassing the BH3 domain. In particular, highly conserved amino acids of the BH3 domain of the pro-apoptotic members of the Bcl-2 family undergo a change in orientation upon cleavage of Bax (Leu-59 and Leu-63). Mutations in these two amino acid residues in a Bak-BH3 peptide have been demonstrated to result in loss of binding capacity to Bcl-x_L (59). Moreover, the changes in the molecular orientation of Leu-59 (corresponds to Leu-27 in truncated Bax) and Leu-63 (Leu-31) were accompanied by a conformational change of Glu-61 (Glu-29), which is believed to support the interaction of Bax with anti-apoptotic

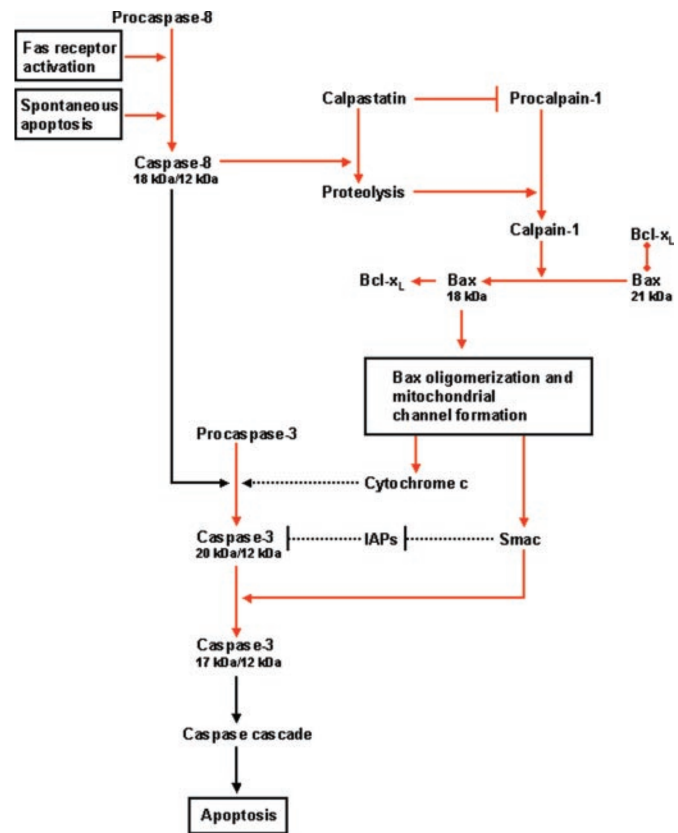

FIG. 8. **A proposed model of the signaling pathways involved in spontaneous and Fas receptor-mediated neutrophil apoptosis.** Activation of the calpastatin-calpain-1 system is an essential proximal event, leading to Bax cleavage and mitochondrial activation. Mitochondrial Smac release appears to be required for neutralizing putative IAPs, which may block autoprocessing of the 20-kDa fragment of caspase-3. Cleavage of procaspase-3 into its 20-kDa form is calpain-independent and might be the result of direct cleavage by caspase-8. It is possible that caspase-9, activated by cytochrome *c* release into the cytosol, also contributes in this process. Furthermore, based on inhibitor studies, effector caspases may further degrade calpastatin. This study provides either direct or indirect evidence for the pathways indicated in *red*.

Bcl-2 family members (60). These data suggested that complete removal of the NH_2-terminal part of the protein in truncated Bax results in a potential inability of this molecule to interact with anti-apoptotic members of the Bcl-2 family and would therefore promote apoptosis.

The theoretical findings of the molecular modeling study were supported by the failure of truncated Bax, in contrast to full-length Bax, to physically interact with Bcl-x_L, as revealed by *in vitro* and *in vivo* co-immunoprecipitation experiments. Cleavage of Bax and its potential liberation from Bcl-x_L suggested that calpain regulates a mitochondria-dependent apoptosis pathway in neutrophils. Earlier work suggested that the role of mitochondria in such a death pathway is the release of cytochrome *c*, which activates with the help of APAF-1 in the presence of dATP the initiator caspase-9 (61). In this scenario, caspase-9 is believed to activate caspase-3. In agreement with this model, we also observed that cytochrome *c* is released from mitochondria in the process of spontaneous and Fas receptor-mediated apoptosis and this was blocked by calpain inhibition.

Our results of the experiments on caspase-3 processing, however, caused some doubt whether the pathway via cytochrome *c*-APAF-1-caspase-9 would be indeed sufficient for caspase-3 activation in neutrophils. For instance, we observed that calpain inhibition results in accumulation of a 20-kDa fragment of caspase-3, which is, because cytochrome *c* is not released under

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these conditions, most likely the consequence of direct caspase-8 action on procaspase-3 (49). Interestingly, the 20-kDa fragment was not enzymatically active, suggesting inhibition of its autoprocessing by one or several IAPs (62). Because we have not seen at any time such an accumulation of 20-kDa caspase-3 in the absence of calpain inhibition, it is unlikely that higher levels of this product can be generated via cytochrome *c*-APAF-1-caspase-9 that could overcome the inhibitory effect of the putative IAP(s). In cell-free extracts of neutrophils, however, a role of cytochrome *c* in processing of caspase-3 has been suggested (63).

We therefore investigated the mitochondrial release of Smac, which activates caspases by inhibition of IAPs (50, 51). In addition to cytochrome *c*, mitochondria released at the same time Smac in both spontaneous and Fas receptor-mediated neutrophil apoptosis, and direct delivery of a heptapeptide, containing the minimal IAP-binding motif of Smac (29), induced death in these cells. Calpain-inhibition blocked Smac release from mitochondria, but this pharmacological intervention no longer prevented death when Smac was artificially delivered into the cytosol. Moreover, addition of Smac to E64-d-treated neutrophils resulted in the occurrence of the 17-kDa fragment of caspase-3, and this was accompanied by strong increases in DEVDase activity. Together these data suggest that 1) one or more IAPs inhibit autoprocessing of the 20-kDa caspase-3 fragment as long as mitochondria are not activated, and 2) blocking of IAPs by cytosolic Smac is a critical pro-apoptotic event in neutrophils that is regulated by calpain-1. The IAP(s) expressed in neutrophils that suppress caspase-3 remains to be investigated, but may involve survivin (data not shown).

The newly described calpain-1-Bax-Smac-caspase-3 pathway (Fig. 8) may help explain why different cell types of cytochrome $c^{-/-}$, APAF-$1^{-/-}$, and caspase $9^{-/-}$ mice demonstrate normal Fas receptor-mediated apoptosis (58). In contrast to previous interpretations, we believe that the role of mitochondria in Fas receptor-mediated apoptosis is not excluded by these findings because the possibility exists that Smac release is sufficient to activate caspase-3 in these genetically modified cells. In neutrophils, in which the presence of mitochondria has been negated (64), we obtained clear evidence that both the spontaneous as well as the Fas receptor-mediated apoptosis requires an intact mitochondrial pathway (65). Although this would be consistent with a model suggesting that spontaneous apoptosis is induced by Fas ligand/Fas receptor interactions (66), recent work suggests that this is not the case (8, 67).

This raises the question regarding the initial trigger of spontaneous neutrophil apoptosis. Although this study does not answer the question, it appears to be clear that the proteolysis of calpastatin, the endogenous inhibitor of calpain (27, 28), is an early event. Exogenous delivery of a peptide that reconstitutes calpastatin activity blocked spontaneous and Fas receptor-mediated death of neutrophils, giving independent confirmation that abolishment of the calpastatin inhibitory activity on calpains is a crucial event leading to execution of apoptosis in neutrophils. This model is also supported by the observation that selective reduction of calpastatin by antisense oligodeoxynucleotides has been shown to accelerate spontaneous apoptosis of neutrophils (68) and by the observation that neutrophils from patients suffering from cystic fibrosis have up-regulated calpastatin and decreased calpain-1 protein levels associated with delayed spontaneous apoptosis. Although the factors and molecular mechanisms by which calpain-1 and calpastatin levels are regulated remain to be established, our data clearly suggest an important proximal role of these two molecules in regulating a new pathway, which is mitochondria-dependent and controls neutrophil apoptosis under normal and inflammatory conditions. Pharmacological targeting of the calpastatin-calpain system in neutrophils might be a promising therapeutic strategy in diseases in which their numbers are dysregulated.

Acknowledgments—We are indebted to Thomas Brunner for reagents and critical discussions of the work; John S. Elce for calpain-1 antibody; James C. Powers for calpain-isotype specific inhibitors; Peter Krammer for CEM, SKW6.4, and H9 cell lines; Jean-Claude Martinou for the plasmid containing Bax cDNA; Lawrence H. Boise for the Bcl-x plasmid; and S. Martin for the HEK 293T cells overexpressing Smac. We also thank Dagmar Simon and Stephan Russmann for providing blood samples from normal donors, Carmen Cassaulta for blood samples from cystic fibrosis patients, and Inés Schmid for analysis of caspase-3-like enzymatic activities.

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J. Biol. Chem. 2004, 279:5947-5957.
doi: 10.1074/jbc.M308576200 originally published online November 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308576200

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