Cathepsin D Primes Caspase-8 Activation by Multiple Intra-chain Proteolysis*

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Background: The exact mechanism of caspase-8 activation by cathepsin D remains unclear.

Results: The generation of an active caspase-8 requires both cathepsin D-mediated proteolysis and homodimerization of caspase-8.

Conclusion: Cathepsin D is able to directly activate caspase-8.

Significance: Cathepsin D-induced caspase-8 activation may represent a general mechanism to induce apoptosis in the absence of death receptor activation in a variety of immune and nonimmune cells.

During the resolution of inflammatory responses, neutrophils rapidly undergo apoptosis. A direct and fast activation of caspase-8 by cathepsin D was shown to be crucial in the initial steps of neutrophil apoptosis. Nevertheless, the activation mechanism of caspase-8 remains unclear. Here, by using site-specific mutants of caspase-8, we show that both cathepsin D-mediated proteolysis and homodimerization of caspase-8 are necessary to generate an active caspase-8. At acidic pH, cathepsin D specifically cleaved caspase-8 but not the initiator caspase-9 or -10 and significantly increased caspase-8 activity in dimerizing conditions. These events were completely abolished by pepstatin A, a pharmacological inhibitor of cathepsin D. The cathepsin D intra-chain proteolysis greatly stabilized the active site of caspase-8. Moreover, the main caspase-8 fragment generated by cathepsin D cleavage could be affinity-labeled with the active site probe biotin-VAD-fluoromethyl ketone, suggesting that this fragment is enzymatically active. Importantly, in an in vitro cell-free assay, the addition of recombinant human caspase-8 protein, pre-cleaved by cathepsin D, was followed by caspase-3 activation. Our data therefore indicate that cathepsin D is able to initiate the caspase cascade by direct activation of caspase-8. As cathepsin D is ubiquitously expressed, this may represent a general mechanism to induce apoptosis in a variety of immune and nonimmune cells.

Apoptosis is a key mechanism in the build up and maintenance of innate and adaptive immunity as well as in the regulation of cellular homeostasis. Multicellular organisms use this apoptotic process to eliminate unwanted and potentially harmful cells from inflamed tissues without releasing hazardous intracellular contents (1–3). Apoptosis is the most common form of physiological cell death (4, 5). The central component of apoptosis is a proteolytic amplifying cascade involving specialized proteases called caspases (6). Caspases are a family of evolutionarily conserved cysteinyl proteases mediating initiation and execution of apoptosis through aspartate-specific cleavage of a wide number of cellular substrates (7). Caspases are organized into an N-terminal domain followed by a catalytic domain usually containing a large subunit of about 20 kDa and a small subunit of about 10 kDa (see Fig. 1A) (8). In a number of procaspases, the large and small subunits are separated by a small linker sequence. Typically, caspases are divided into three major groups based on the structure of the prodomain and their function; caspases with large prodomains are referred to as inflammatory caspases (caspases-1, -4, -5, -11, -12, -13, and -14) and initiator of apoptosis caspases (caspases-2, -8, -9, and -10), whereas caspases with short prodomains, containing 20–30 amino acids, serve as effector caspases (e.g. caspases-3, -6, and -7) (9).

Work using recombinant caspases and in vitro conditions hypothesized that initiator caspases are activated by dimerization but not by intra-chain proteolysis (reviewed in Refs. 10, 11). Intra-chain proteolysis was, in fact, shown to substantially stabilize the active form of caspase-8 (8). Nevertheless, some authors disputed this and showed that intra-chain proteolysis by granzyme B or caspase-6, in the absence of enforced homodimerization of caspase-8 by Fas-associated protein with death domain, was able to activate caspase-8 in vitro (12, 13). Because of this controversy, additional studies were performed using in vivo deletion of mouse caspase-8 (14), in vitro reconstitution of a human DISC2 (15), and cellular reconstitution of human caspase-8 (16, 17). These studies show that robust apoptosis is dependent on a two-step process whereby homodimerization of caspase-8 is followed by (auto)processing to generate an apoptosis-promoting enzyme.

Beside caspases, other proteases have been shown to be involved in cell death regulation. Among them, the lysosomal cathespins seem to play a crucial role. Cathespins were shown to process Bcl-2 family members, such as Bid and Bax, and also proinflammatory procaspase-1 and procaspase-11 as well as...
the initiator procaspase-2 (18–23). During spontaneous neutrophil apoptosis, cathepsin D is rapidly released from azurophilic granules in a caspase-independent but reactive oxygen species-dependent manner, leading to a direct and death receptor-independent activation of the initiator caspase-8, which then can activate caspase-3 and launch the full apoptotic machinery (24).

Direct activation of caspases by cathepsins may therefore have physiological relevance, at least in neutrophils. Nevertheless, the exact mechanism remains to be elucidated. In particular, we were interested in the potential role of the intra-chain proteolysis of caspase-8 in its activation. To address this question, we generated site-specific mutants of caspase-8. Our data suggest a new activation mechanism of caspase-8 in which both cathepsin D-induced proteolysis of the initiator caspase-8 and its homodimerization are necessary to launch an efficient apoptotic program in neutrophils and, potentially, in a variety of other immune and nonimmune cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cathepsin D recombinant enzyme was obtained from Merck. The specific fluorogenic cathepsin D substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-2,4-dinitrophenyl)-l-2,3-diamino propionyl-Ala-Arg-NH₂) and pepstatin A (PepA) were from R & D Systems Europe Ltd. (Abingdon, UK) and Sigma, respectively. The fluorogenic substrates of caspase-8/10 (Ac-IETD-AFC) and caspase-9 (Ac-LEHD-AFC) as well as b-VAD-FMK were purchased by MP Biomedicals (Solon, Ohio).

**Cells**—Mature blood neutrophils were isolated from peripheral blood of healthy donors by Ficoll-Hypaque centrifugation (24, 25). The resulting cell populations contained greater than 95% mature neutrophils as assessed by staining with Diff-Quik (Medion GmbH, Düdingen, Switzerland) and light microscopy analysis. All individuals who donated blood signed a written informed consent agreement in accordance with the Declaration of Helsinki. The study was approved by the local medical ethics committee (the Kantonale Ethikkommission Bern).

**Cell Cultures**—Human blood neutrophils were cultured at 10⁶ cells/ml in complete culture medium (RPMI 1640 containing 10% fetal calf serum) for the indicated times.

**Preparation of Cytosolic Extracts**—50 × 10⁶ freshly isolated human mature blood neutrophils were washed with cold PBS and lysed for 15 min on ice in 150 μl of CEB buffer (250 mM sucrose, 70 mM KCl, 250 μg/ml digitonin, 2 mM PMSF, 5 mM sodium orthovanadate supplemented with protease inhibitor mix-
Caspase-D-mediated Caspase-8 Activation

The activity of caspases was carried out as described previously (28). The uncleaved caspase-8 (Casp8-D2A, EA), or the uncleaved caspase-10 (Casp10-DA) containing D372A mutation) mutants were cloned as cleavable caspase-10 (caspase-10 lacking the first 202 residues; Casp10-DA) and noncleavable caspase-8 (Casp8-D2A, EA) containing D315A, D330A, and E306A mutations, and DED inactive caspase-8 (Casp8-CA) harboring D374A mutations, DED noncleavable caspase-8 (Casp8-D2A) harboring D374A and D384A mutations, DED active caspase-8 (Casp8-CA), noncleavable caspase-9 (Casp9-D2A, EA) containing D315A, D330A, and E306A mutations, and DED noncleavable caspase-10 (caspase-10 lacking the first 202 residues; Casp10-DA containing D372A (Casp10-DA) mutation) mutants were cloned as described previously (26, 27). All mutations were generated by overlapping PCR. All plasmids were sequenced, and only error-free plasmids were used in this study. Expression and purification of caspases were carried out as described previously (28).

Activity Measurements and Separation on SDS Gels—Caspase-D activity was measured using pure caspase-D enzyme as enzymatic conversion of the fluorogenic cathepsin D substrate after 30 min of incubation at 37 °C according to the manufacturer’s instructions. The cathepsin D enzyme was diluted in one of the following assay buffers: 50 mM glycine, 100 mM NaCl, pH 3.0; 50 mM glycine, 100 mM NaCl, pH 3.5; 50 mM sodium citrate, 100 mM NaCl, pH 4.0; 50 mM sodium citrate, 100 mM NaCl, pH 4.5; 50 mM sodium citrate, 100 mM NaCl, pH 5.0; 50 mM MES, 100 mM NaCl, pH 6.0; PBS, pH 7.4.

For determination of the enzymatic activity, 0.3 units of pure caspase-D enzyme, the uncleaved caspase-8 (Casp8-D2A), wild-type caspase-8 (Casp8-WT), the uncleaved caspase-9 (Casp9-D2A, EA), or the uncleaved caspase-10 (Casp10-DA) were diluted in the indicated assay buffers or PBS, pH 7.4, at final concentrations of 2 μM and incubated for 30 min at 37 °C with or without the indicated amount of cathepsin D supplemented or not with PepA (0.25 μM). The samples were then diluted in low salt buffer (20 mM PIPES, pH 7.5, 100 mM NaCl, 0.05% CHAPS, 10 mM DTT, and 5% sucrose) or high salt buffer (same as above but containing 1 mM sodium citrate) at final concentrations of 2–100 nM and incubated for 15 or 30 min at 37 °C. Caspase-8/caspase-10 and caspase-9 activities were measured by adding the substrates Ac-IETD-AFC and Ac-LEHD-AFC (100 μM if not indicated). To check the processing of caspase-8/caspase-9 and caspase-10, samples were also analyzed by 8–18% SDS-PAGE and revealed by Coomassie staining (29).

Caspase-3 activity was measured in 15 μg of cytosolic extracts prepared from freshly isolated mature neutrophils supplemented with the indicated final concentrations of Casp8-WT or Casp8-D2A (ratio between the volumes of neutrophil extracts and casp-8; from 4:1 to 1:1), preincubated for 15 min at 37 °C with or without 0.3 units of caspase-D enzyme in assay buffer, pH 5.0, followed by artificial homodimerization for 30 min at 37 °C in high salt buffer. A commercial caspase-3 cellular activity assay kit (Quantzyme, BIOMOL International, L.P.), containing the substrate Ac-DEVD-p-nitroaniline, was used according to the manufacturer’s instructions.

Labeling of Active Caspase-8—To label the active sites of caspase-8, the uncleaved caspase-8 (Casp8-D2A) was diluted in assay buffer, pH 5.0, at final concentrations of 2 μM and incubated for 30 min at 37 °C with the indicated amount of cathepsin D. The samples were then diluted in high salt or low salt buffer at final concentrations of 80 nM and incubated for 30 min at 37 °C. Activated caspase-8 was also detected in 15 μg of cytosolic extracts from freshly isolated human blood neutrophils, which were incubated in the presence or absence of 0.3 units of caspase D and PepA (1 μM) for 30 min at 37 °C. 1 μM biotin-VAD-FMK was added, and samples were incubated for 15 min at RT. Proteins were precipitated with TCA, dissolved in SDS sample buffer, and analyzed by SDS-PAGE. Separated proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA). The filters were blocked overnight at RT in TBS containing 0.1% Tween 20 and 2% BSA, washed for 1 h at RT in TBS containing 0.4% Tween 20, and incubated for 30 min at RT in TBS containing 0.1% Tween 20 and 2% BSA with avidin-horseradish peroxidase-conjugated antibody (1:5000; Thermo Fisher Scientific). Filters were developed by an enhanced chemiluminescence technique (ECL kit; GE Healthcare) according to the manufacturer’s instructions.

Immunoblotting—106 cells were washed with cold PBS supplemented with protease mixture inhibitor and lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA supplemented with protease inhibitor mixture). After centrifugation to remove insoluble particles, equal amounts of cell lysates or 15 μg of cytosolic extracts from freshly isolated human blood neutrophils incubated in the presence or absence of 0.3 units of caspase-D and PepA (1 μM) for 30 min at 37 °C were loaded on gels (NuPAGE, Invitrogen). Separated proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The filters were incubated overnight at 4 °C in TBS, 0.1% Tween 20, 5% nonfat dry milk with mouse monoclonal anti-caspase-8 antibody (1:1000, clone 12F5, Enzo Life Sciences AG, Lausen, Switzerland). Filters were washed in TBS, 0.1% Tween 20, 5% nonfat dry milk for 30 min at RT and incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (GE Healthcare) in TBS, 0.1% Tween 20, 5% nonfat dry milk for 1 h. Filters were developed by an enhanced chemiluminescence technique (ECL kit; GE Healthcare) according to the manufacturer’s instructions.

N-terminal Sequencing of Protein Samples—Protein samples were resolved by SDS-PAGE and transferred to a PVDF membrane by electroblotting, and the membrane was briefly stained in Coomassie Brilliant Blue R-250. Appropriate bands were excised and sequenced by Edman degradation.

Statistical Analysis—Statistical analysis was performed with the Mann-Whitney U test. If mean levels are presented, the means ± S.D. and the number (n) of independent experiments are indicated in each case. A probability value of <0.05 was considered statistically significant.

RESULTS

Caspase-D-mediated Cleavage of Caspase-8 Followed by Its Homodimerization Significantly Increases Caspase-8 Activity—Caspase-D is a protease that critically depends on protonation of its active site Asp residues (30). Along with Asp protonation,
Acidic pH also leads to an important conformational change in cathepsin D moving the N-terminal segment of the protease out of the active site (31). Therefore, above a certain pH, cathepsin D could be deprotonated and inactivated. To test the highest limit of pH necessary to obtain an active cathepsin D, we performed an in vitro experiment using pure recombinant cathepsin D enzyme. We observed detectable enzymatic activities at pH 5.0 and lower (see Fig. 2A). As expected, at higher pH, almost no cathepsin D activity was detected within the constraints of this assay, arguing that cathepsin D needs lysosomal pH to be active.

In vitro, cathepsin D was shown to directly cleave caspase-8 at pH 3.0 as well as in neutrophil cytosolic extracts (24). We decided to check whether at pH 5.0 cathepsin D was able to process and activate caspase-8 by using site-specific mutants that can be activated in a controlled manner (see Fig. 1C). Because recombinant full-length caspase-8 was insoluble in our hands, we used caspase-8 constructs (mainly Casp8-WT and Casp8-D2A) from which the N-terminal death effector domains (DEDs) were deleted (see Fig. 1, A and B). Because of autoproteolysis during Escherichia coli expression, recombinant wild-type caspase-8 (Casp8-WT) prepared without DEDs generated a two-chain species that occurs as a mixture of inactive monomer and active dimer, with a $K_d$ value in the low micromolar range (8, 26, 32). A single-chain mutant (Casp8-D2A) that cannot be autoprocessed is a stable inactive monomer (8). Addition of artificial dimerizing conditions, like high salt kosmotropic buffers, changes the equilibrium to an active dimer without the need for cleavage, reflecting the likely mechanism of activation at the DISC (32–34). At pH 5.0 and lower, we obtained evidence that the addition of cathepsin D was followed by cleavage of the single-chain caspase-8 linker mutants into several fragments (see Figs. 1 and 2B). Activity measurements at neutral pH confirmed that cathepsin D significantly increased the enzymatic activity of the single-chain mutant, Casp8-D2A, when cleavage of 30 min was followed by artificial homodimerization (see Fig. 2C). Moreover, the enhanced caspase-8 activity was more pronounced after 30 min of incubation with the kosmotropic buffer than after 15 min (the ratio between 5604 ± 2805 in the presence of cathepsin D and 243 ± 117 for buffer, pH 5.0, results in a 23-fold increase after 30 min and the ratio between 17100 ± 6336 in the presence of cathepsin D and 1476 ± 936 for buffer, pH 5.0, results in a 11-fold increase after 15 min). Importantly, the caspase-8 substrate Ac-IETD-AFC could not be cleaved by cathepsin D (Fig. 1C). Together, our data indicate that cathepsin D was able to directly process caspase-8 at pH 5.0, which is approximately the pH found in lysosomes, resulting in significant increase of activity of the dimerized caspase-8 at neutral pH.

Sequentially by Edman degradation revealed that cathepsin D cleaves caspase-8 at Leu$^{237}$ in the large subunit (e.g. confirming our identified cleavage site on caspase-8, see Ref. 24), at Met$^{383}$ in the intersubunit linker, and at Leu$^{443}$ in the small subunit (see Fig. 1B). The relative molecular mass of these fragments, deduced from the ExPASy software, was 21.2 kDa (band 1, fragment Ile$^{238}$–Met$^{383}$), 6.8 kDa (band 2, fragment Ala$^{384}$–Leu$^{443}$), 4.8 kDa (band 3, fragment start-Leu$^{237}$), and 4.2 kDa (band 4, fragment Thr$^{444}$-end). Interestingly, we could detect the same fragments by incubating cathepsin D with the inactive Casp8-CA mutant (see Fig. 1A) indicating that the cathepsin D cleavages were conserved regardless of the caspase-8 activity status (Fig. 3A). The generation of multiple cleavages of caspase-8 is in agreement with the view that cathepsins cleave their substrate in a sequence-unspecific manner, presumably governed by structural constraints (6).

Cathepsin D Specifically Primes Caspase-8 for Activation—Because cleavage by cathepsin D fulfilled one requirement for caspase-8 efficient activation, we addressed the question of whether a pharmacological inhibitor of cathepsin D could block this caspase-8 activation and whether cathepsin D could act on other initiator caspases, such as caspase-9 and -10. As shown in Fig. 3, the cathepsin D inhibitor PepA blocked both the processing of the single-chain mutant, Casp8-D2A (see Fig. 3A) and, consequently, the increase in high salt enzymatic activity (see Fig. 3B) of the same mutant. In contrast, under the same conditions, cathepsin D was unable to cleave and change the enzymatic activity of neither the single-chain mutant of caspase-10 (Casp10-DA, see Fig. 3, C and D) nor of caspase-9 (Casp9-D2A, EA, see Fig. 3, E and F). Taken together, our results
indicate that cathepsin D-mediated cleavage can specifically prime caspase-8 for activation but has no consequence on the ability of initiator caspase-9 and -10 to become active.

**Cathepsin D Cleavage of Monomeric Caspase-8 Is Insufficient for Activation**—As cathepsins were shown to cleave their substrate in a relatively unspecific manner (6), point mutations of the different cleavage sites would be uninformative regarding the activation mechanism of caspase-8 by cathepsin D. Consequently, we performed a concentration-dependent cleavage of the single-chain mutant of caspase-8 by cathepsin D to carefully follow the kinetics of caspase-8 processing. Cathepsin D concentration-dependently increased the enzymatic activity of the single-chain mutant of caspase-8 by cathepsin D to carefully follow the kinetics of caspase-8 processing. Cathepsin D concentration-dependently increased the enzymatic activity of the single-chain mutant of caspase-8 only when homodimerization followed cleavage (see Fig. 4A). To sequentially check the processing of caspase-8 by cathepsin D, we used the same *in vitro* samples, as in the Fig. 4A, and separated them on SDS gels. Coomassie staining shows that band 1 corresponding to the 21.2-kDa fragment (see also Fig. 2B) was first formed with a low concentration of cathepsin D (0.0094 units), indicating that the primary cleavages occur at Leu237 and Met383 in the large subunit and intersubunit linker, respectively (see Fig. 4B). After this, the bands 2–4 (see also Fig. 2B) seem to appear simultaneously, arguing that once the first cleavages have occurred, a further processing of caspase-8, with a cleavage at Leu443 in the small subunit, occurs relatively quickly.

Dimerizing conditions, like high salt kosmotropic buffers, form an active dimer of the single-chain caspase-8 mutant (Casp8-D2A) without the need for cleavage *in vitro*. To challenge the hypothesis that cleavage of caspase-8 by cathepsin D would be sufficient to generate an active form of caspase-8, we incubated *in vitro* the single-chain mutant of caspase-8 with cathepsin D and followed the enzymatic activity of caspase-8 in nondimerizing conditions. In accordance with previous reports (8, 10, 11), these conditions did not produce active species of the single-chain mutant of caspase-8 following intra-chain proteolysis, regardless of the amount of cathepsin D used (Fig. 4A, upper panel). Therefore, our data indicate that homodimerization of caspase-8 is a prerequisite step to obtain an active enzyme. Cathepsin D-mediated intra-chain proteolysis likely provides efficient organization of caspase-8 active site loops.

The activity-based probe biotin-Val-Ala-Asp-fluoromethyl ketone (b-VAD-FMK) reacts preferentially with the active form of caspases. Therefore, we decided to check which caspase-8 fragments were enzymatically active under dimerizing (*e.g.* high salt) and nondimerizing (low salt) conditions (see Fig. 4C). In an *in vitro* assay using the uncleaved caspase-8 (Casp8-D2A), we...
observed that the addition of cathepsin D generated an active fragment with a molecular mass of about 21 kDa (e.g. corresponding to the band 1 of the Fig. 2B), which was labeled by b-VAD-FMK during dimerizing but not during nondimerizing assay conditions (Fig. 4C). In addition, in high salt buffer, the single-chain, unproteolyzed Casp8-D2A was also labeled by b-VAD-FMK (31-kDa band), a confirmation of its basal activity prior to processing (see Fig. 4C, upper panel, top band). In conclusion, our data confirm that homodimerization and limited proteolysis of the inter-subunit linker of caspase-8 to a 21-kDa fragment are necessary to generate high activity in this initiator caspase.

**Cathepsin D Improves the Catalytic Parameters of Caspase-8**—Next, we tested how cathepsin D cleavage modifies the catalytic parameters of the single-chain mutant of caspase-8 (Casp8-D2A) (see Fig. 5). The enzymatic activity of the mutant Casp8-D2A was measured in high salt buffer at various concentrations of the specific substrate Ac-IETD-AFC. In agreement with our previous experiments, caspase D was able to increase the enzymatic activity of caspase-8 in a dose-dependent manner as shown by a progressive increase of \( v_{\text{max}} \). This suggests that the cathepsin D-induced proteolysis causes the active site of caspase-8 to accommodate the substrate in a more efficient manner.

To quantify the degree of change in activity upon caspase-8 cleavage, we compared the catalytic parameters of two mutants cleaved by cathepsin D, single-chain (Casp8-D2A) and two-chain (Casp8-WT) (see Table 1). Although the \( K_{\text{m}} \) value was generally decreased by the presence of cathepsin D, \( k_{\text{cat}} \) was increased about 3–15 times for Casp8-D2A and about 2-fold for Casp8-WT. The highest increase of \( k_{\text{cat}} \) was achieved with the highest concentration (0.3 units) of cathepsin D used. Consequently, the total increase in the high salt activity upon exposure to cathepsin D was 100-fold for Casp8-D2A and about 2-fold for Casp8-WT, generating two species of comparable kinetic parameters (see Table 1). Overall, the results demonstrate that proteolysis by cathepsin D increases both the affinity of dimeric caspase-8 for its substrate as well as its catalytic efficiency.

**Cathepsin D-activated Caspase-8 Activates Caspase-3 in Neutrophil Cell-free Extracts**—To determine whether the cathepsin D proteolysis resulting in caspase-8 activation is relevant in a more physiological context, we performed *in vitro* cell-free assays using cytosolic extracts of freshly isolated blood neutrophils. We first tested whether cathepsin D was able to efficiently process caspase-8 in our cell-free assays. As shown previously (24), we observed that the addition of cathepsin D was followed by the cleavage of caspase-8 (15-kDa fragment), which was not seen in the presence of pepstatin A (Fig. 6A, left panel). In addition, the caspase-8 fragment generated by incubation of cytosolic fractions with cathepsin D could be affinity-labeled with the biotinylated caspase substrate VAD-FMK (b-VAD-FMK; Fig. 6A, middle panel), suggesting that the 15-kDa fragment of caspase-8 is enzymatically active. Importantly and in agreement with our previously published work (24, 35), we could observe a similar caspase-8 cleavage in association with spontaneous neutrophil apoptosis (Fig. 6A, right panel). As shown previously (24), this cleavage was completely inhibited by the presence of PepA. These data suggest that cathepsin D is able to efficiently activate caspase-8 during physiological apoptosis of neutrophils.

After an incubation of the wild-type caspase-8 (Casp8-WT) or the single-chain mutant of caspase-8 (Casp8-D2A) with or without cathepsin D, we added these mixtures to neutrophil cytosolic extracts and followed the enzymatic activity of caspase-3 (see Fig. 6B). The addition of caspase-8 that had been exposed to cathepsin D and artificially dimerized caused induction of caspase-3 activity in cytosolic extracts of neutrophils in a more efficient manner than the wild-type caspase-8 or cathepsin D alone (24). In contrast, the addition of caspase-8 not cleaved by cathepsin D or cleaved by cathepsin D but not artificially dimerized did not induce caspase-3 activation in the same conditions (see Fig. 6B). Importantly, cathepsin D was not able to directly cleave and activate caspase-3 (shown in Ref. 24) and PepA abolished, in the same conditions, caspase-3 activation by cathepsin D-activated caspase-8 (Fig. 6B). Taken together, our data confirm that both homodimerization and proteolysis of caspase-8 by cathepsin D were required to efficiently activate caspase-8, which then might be able to induce apoptosis in a more physiological system such as neutrophils.
Cathepsin D-mediated Caspase-8 Activation

FIGURE 5. Cathepsin D increases caspase-8 activity in a concentration-dependent manner. Activity of uncleavable caspase-8 mutant Casp8-D2A incubated with the indicated amount of cathepsin D as a function of the concentration of caspase-8 substrate, Ac-IETD-AFC, measured upon equilibration for 30 min in high salt assay buffer. The results are representative of three independent experiments. The catalytic parameters of these reactions are shown in Table 1.

TABLE 1
Catalytic parameters of Casp8-WT and Casp8-D2A
Casp8-WT and Casp8-D2A were incubated with the indicated amounts of cathepsin D. Measurements were performed in high salt assay buffer at 37 °C against the artificial substrate Ac-IETD-AFC.

| Mutant            | Cathepsin D concentration | $k_{cat}$ (units) | $k_{cat}/K_m$ | $K_m$ (μM) |
|-------------------|---------------------------|-------------------|---------------|-------------|
| Casp8-D2A         | 0.019                     | 4.9 ± 0.7         | 0.014 ± 0.002 | 2.9 × 10²   |
| Casp8-D2A         | 0.075                     | 4.3 ± 1.7         | 0.010 ± 0.002 | 2.2 × 10³   |
| Casp8-D2A         | 0.3                       | 5.1 ± 0.7         | 0.060 ± 0.020 | 1.2 × 10⁴   |
| Casp8-D2A         | 16.3                      | 3.4 ± 0.8         | 0.080 ± 0.017 | 2.4 × 10⁶   |
| Casp8-D2A         | 4.1                       | 4.1 ± 0.3         | 0.146 ± 0.015 | 3.6 × 10⁶   |

DISCUSSION

The central component of apoptosis is a family of intracellular proteases with aspartate specificity, called caspases. However, at least in neutrophils, the initial events leading to caspase activation have remained obscure. Beside caspases, it has been proposed that non-caspase proteases can play a crucial role in the regulation of apoptosis in several cell types, including neutrophils (35, 36). Surprisingly, we found that the aspartic lysosomal protease, cathepsin D, acts proximal to caspases and directly activates the initiator caspase-8 to launch spontaneous neutrophil apoptosis (24). Nevertheless, the exact mechanism of caspase-8 activation by cathepsin D remained to be elucidated. In this paper, we provide evidence that, during neutrophil apoptosis, intra-chain proteolysis at both Leu237 and Met383 of caspase-8 by cathepsin D is not sufficient to activate caspase-8 but seems to more efficiently organize the active site of caspase-8 following its homodimerization. It is important to note that the sequential activation of caspase-8 may differ in a stimulus- and cell type-dependent manner.

As neutrophils from Fas receptor- or Fas ligand-deficient mice die normally in vitro (37), the entry into spontaneous neutrophil apoptosis was thought to be independent of death receptor signaling but regulated by the intrinsic pathway. Nevertheless, spontaneous apoptosis of neutrophils requires the activity of caspase-8 (24, 35), an enzyme that is normally activated downstream of death receptor activation. Thus, how does caspase-8 form homodimers in neutrophils? An interesting possibility is that the activation platform of caspase-8 is the phospholipid cardiolipin. Gonzalvez et al. (38) showed a novel role for the mitochondrial cardiolipin in the activation of the apical caspase-8 in type II cells, which requires the mitochondrial amplificatory loop. Following an external stimulus, caspase-8 translocates to the mitochondrial membrane where it binds to cardiolipin. This will induce oligomerization and cathepsin D-mediated cleavages of caspase-8 and its homodimerization are absolutely required to obtain a maximally active enzyme suitable for apoptotic processes in vitro and in vivo (8, 16, 31, 34).

Our previous report demonstrated detectable caspase-8 activity in neutrophil cytosolic extracts supplemented with cathepsin D recombinant enzyme (24). As an active monomeric caspase has not been described yet, we speculate that the modest activity of cathepsin D-cleaved caspase-8 rises from a fraction of dimer favorably generated post-cleavage. Indeed, it has been previously shown that caspase-8 linker proteolysis dramatically increases the barrier for caspase-8 homodimerization in comparison with the unprocessed form (8, 32). We also cannot rule out that monomer association could be favored by the slight acidic pH or by the neo-epitopes produced by cathepsin D cleavage versus the natural neo-epitopes produced by autocleavage. Using b-VAD-FMK, we demonstrated that the cleaved form of caspase-8 was active (see Fig. 4C) and that active caspase-8 processed caspase-3 launching apoptosis in physiological conditions such as in neutrophils (see Fig. 6). However, without homodimerization, intra-chain proteolysis of caspase-8 by cathepsin D is not sufficient to activate caspase-8 but seems to more efficiently organize the active site of caspase-8 following its homodimerization. It is important to note that the sequential activation of caspase-8 may differ in a stimulus- and cell type-dependent manner.
activation of caspase-8 to launch the apoptotic machinery. In this model, caspase-8 was only partially inserted into the outer mitochondrial membrane and was still accessible from the cytosol arguing that, once released from azurophilic granules, cathepsin D could directly cleave caspase-8 and very efficiently launch neutrophil apoptosis. A second possibility, which would better fit with our results, is that cathepsin D-cleaved caspase-8 would directly form homodimers on the membrane of azurophilic granules. In this respect, it is interesting to note that caspase-8 was found to be recruited by autophagy-related gene 5 to autophagosomal membranes (39). Clearly, further investigations need to be performed to identify the activation platform of caspase-8 in neutrophils.

Our data indicate that cathepsin D needs an acidic pH, corresponding to the pH found in lysosomes, to efficiently activate caspase-8 (see Fig. 2). Indeed, cathepsin D was shown to be inactivated at the cytoplasmic pH (40). Therefore, how can cathepsin D activate caspase-8 within a cell? One possible explanation is that cathepsin D cleaved caspase-8 via its known endopeptidase activity, which was shown to be retained for a brief interval at neutral pH (41, 42). A second explanation is that acidification of the cytosol may occur, at least in close proximity of lysosomes, due to lysosome membrane permeabilization during apoptosis, allowing cathepsin D to be completely active. This is in accordance with previous reports showing acidification of the cytosol in apoptotic cells (43, 44). The most direct evidence that cathepsin D physiologically maintains its activity in the cytosol is demonstrated by the delayed neutrophil apoptosis in cathepsin D−/− mice (24).

A growing number of cytosolic substrates of lysosomal cathepsins have been reported, and for the most part they are consistent with promoting the apoptotic program (reviewed in Ref. 1). Although we initially reported (24) that cathepsin D is rapidly released from azurophilic granules leading to a direct activation of the initiator caspase-8 by cleavage at Leu237 in the large subunit, it is now clear that this cannot be a complete activating event, but cleavages at both Leu237 and Met383 fit this requirement. Cathepsin D is able to cleave the initiator caspase-8, but not the initiator caspases-9 and -10, arguing that cleavage of caspase-8 by cathepsin D was specific (see Fig. 3). This indicates that once released from azurophilic granules, cathepsin D launches spontaneous neutrophil apoptosis by specifically cleaving the initiator caspase-8. The exact molecular mechanisms responsible for the permeabilization of azurophilic granules remain to be investigated. Nevertheless, permeabilization of azurophilic granules may provide a new therapeutic strategy to induce neutrophil apoptosis and, consequently, to resolve unwanted innate immune responses. Importantly, our in vitro studies, using pure recombinant enzymes, and the finding that cathepsin D is ubiquitously present in lysosomes, suggest that this cathepsin D-caspase-8 pathway could play a crucial role in apoptosis of a variety of immune and non-immune cells expressing both proteases.

FIGURE 6. Cathepsin D-activated caspase-8 induces caspase-3 activation and consequently apoptosis in neutrophils. A, cleavage of caspase-8 by cathepsin D in cytosolic extracts from freshly isolated human blood neutrophils (left panel) and cleavage of caspase-8 in fresh and 9-h-cultured human blood neutrophils (right panel). Cytosolic extracts were also used for affinity labeling with b-VAD-FMK and analyzed by immunoblotting (middle panel). Results are representative of three independent experiments. B, wild-type caspase-8 (Casp8-WT) or the single-chain caspase-8 (Casp8-D2A) was incubated or not with cathepsin D (0.3 units) alone or in combination with pepstatin A (1 μM) for 15 min at 37 °C. These samples were then incubated for 30 min at 37 °C in high salt buffer and added to cytosolic extracts of freshly isolated human blood neutrophils at the indicated final concentrations. Caspase-3 activities were measured in a time-dependent manner. Values are means ± S.D. of three independent experiments.
Cathepsin D-mediated Caspase-8 Activation

From a mechanistic viewpoint, although both single-chain and two-chain forms of caspase-8 are kinetically equivalent for sodium citrate activation via a bimolecular event, the degree of stability of the cleaved dimer is substantially higher than that of the single-chain dimer (8). Presumably, the single-chain dimer contains fewer contacts in the loop bundle that defines part of the dimer interface produced by interactions with the cleaved interchain linker (8, 45). In our dimerizing conditions, $k_{\text{cat}}$ was higher and $K_m$ was lower for the two-chain form as compared with the single-chain form indicating that sodium citrate facilitates a more efficient attack on the substrate and a better recognition of the substrate in the case of the two-chain caspase-8 (see Table 1 and Fig. 5). When cathepsin D cleaved the single-chain caspase-8, the kinetic constants were similar to ones of the two-chain caspase-8, suggesting that the catalytic sites of the two species were similarly organized. Interestingly, when cathepsin D was added to the two-chain caspase-8, a slightly better enzyme was generated (see Table 1). Collectively, our data confirm that proteolysis of the single-chain caspase-8 by cathepsin D primes caspase-8 for subsequent activation by homodimerization, setting the grounds for launching spontaneous neutrophil apoptosis.

Finally, there is an important limitation to the interpretation of our study. All of the results apply to recombinant caspase-8 lacking the tandem DEDs at the N terminus of the natural protein. DEDs recruit caspase-8 to the DISC or bring in close proximity multiple monomers. Although DED cleavage is important for the subsequent release of caspase-8 in the cytosol, we cannot predict how the presence of DEDs would affect activation of caspase-8. Nevertheless, the conclusion of our study is that both caspase-8 cleavage by cathepsin D and homodimerization are necessary to generate catalytic activity. Additionally, the new pathway of caspase-8 activation described in this paper may resolve the issue of how this caspase is activated in the new pathway of caspase-8 activation described in this paper.

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