Cathepsin D Triggers Bax Activation, Resulting in Selective Apoptosis-inducing Factor (AIF) Relocation in T Lymphocytes Entering the Early Commitment Phase to Apoptosis

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Activated human T lymphocytes exposed to apoptotic stimuli targeting mitochondria (i.e. staurosporine), enter an early, caspase-independent phase of commitment to apoptosis characterized by cell shrinkage and peripheral chromatin condensation. We show that during this phase, AIF is selectively released from the intermembrane space of mitochondria, and that Bax undergoes conformational change, relocation to mitochondria, and insertion into the outer mitochondrial membrane, in a Bid-independent manner. We analyzed the subcellular distribution of cathepsins (Cat) B, D, and L, in a search for caspase-independent factors responsible for Bax activation and AIF release. All were translocated from lysosomes to the cytosol, in correlation with limited destabilization of the lysosomes and release of lysosomal molecules in a size selective manner. However, only inhibition of Cat D activity by pepstatin A inhibited the early apoptotic events and delayed cell death, even in the presence of bafilomycin A1, an inhibitor of vacuolar type H+ -ATPase, which inhibits acidification in lysosomes. Small interfering RNA-mediated gene silencing was used to inactivate Cat D, Bax, and AIF gene expression. This allowed us to define a novel sequence of events in which Cat D triggers Bax activation, Bax induces the selective release of mitochondrial AIF, and the latter is responsible for the early apoptotic phenotype.

If activated primary T lymphocytes are exposed to apoptotic stimuli that do not rely on death receptors (such as anti-CD2 and staurosporine), they undergo a definitive sequence of apoptotic events (1, 2). This sequence begins with an early phase of commitment to apoptosis, characterized by cell shrinkage, peripheral chromatin condensation, and the partial efflux of apoptosis-inducing factor (AIF) from mitochondria to the cytosol and the nucleus. No caspase activation is observed and thus, internucleosomal DNA fragmentation does not occur. Cytochrome c is subsequently released from mitochondria, and the inner mitochondrial transmembrane potential (ΔΨm) is simultaneously dissipated. These two events constitute a “point of no-return” coinciding with amplification of the caspase cascade and achievement of the apoptotic phenotype.

AIF is an apoptogenic flavoprotein, which resides in the intermembrane space of mitochondria and is translocated, in a caspase-independent manner, to the cytosol and the nucleus in response to apoptotic stimuli (3, 4). In the nuclei, it stimulates peripheral chromatin condensation and large-scale 50-kb DNA fragmentation (5, 6). The partial release of mitochondrial AIF, but not of cytochrome c, observed in T cells after their commitment to apoptosis, suggests that only limited permeabilization of the outer mitochondrial membrane (OMM) occurs at this time. In neurons treated with camptothecin, AIF release is dependent on Bax, a pro-apoptotic member of the Bcl-2 protein family (7). In Caenorhabditis elegans, the mitochondrial release of WAH-1, a homolog of AIF, is promoted by the BH3-domain protein, EGL-1 (8). We therefore thought that Bax might cause AIF relocation in the cells of our system. Bax is present as an inactive monomer in the cytosol. Upon the induction of apoptosis, it is translocated to mitochondria, and becomes inserted via its C terminus into the OMM, where it forms homo-oligomers, provoking membrane permeabilization (9–12). These events require Bax to undergo a conformational change leading to the exposure of cryptic epitopes at the N terminus of the molecule (10, 13, 14). Formation of the active conformation of Bax can be promoted by Bid, a BH3-only protein, present in its full-length inactive form in the cytosol. Bid is activated by proteolytic cleavage to generate a truncated molecule, tBid, resulting in the release of cytochrome c from mitochondria (14). Although tBid is also translocated from the cytosol to become an integral mitochondrial membrane protein, it can induce formation of the active Bax configuration in the cytosol by direct Bax/Bid interaction (15). There are also Bid-independent pathways of Bax insertion into the mitochondria as demonstrated by the use of Bid−/− mouse embryo fibroblasts treated with anti-TNFα (16). Bax translocation/membrane insertion...
serion and cell death may also occur following pH modifications (17, 18), and enforced dimerization of Bax (11).

The aim of this study was to evaluate the extent of mitochondrial perturbation in activated T lymphocytes entering the caspase-independent phase of commitment to apoptosis, and to identify the upstream molecular effectors responsible. We found that Bax was indeed activated, and this led us to analyze the role of noncaspase proteases in this process. We focused in particular on lysosomal proteases, as shown that these proteases are able to substitute for caspases in a number of apoptotic models (reviewed in Refs. 19–21). Pharmacological inhibition of cathepsins (Cat) and the use of small interfering RNA (siRNA)-mediated gene silencing (22) to target Cat D, Bax, and AIF mRNAs, demonstrated that Cat D, which is translocated from lysosomes to the cytosol upon apoptotic signaling, is the earliest event triggering a rapid change in Bax conformation together with insertion of this protein to the OMM. These events, which are independent of Bid cleavage/translocation, result in mild OMM permeabilization, the effects of which are limited to AIF release. AIF triggers in turn the early apoptotic phenotype induced by staurosporine (STS) in activated T lymphocytes.

**Materials and Methods**

**T Lymphocyte Isolation, Culture Conditions, and Cell Death Induction—**Peripheral blood leukocytes were isolated from blood bank leukopheresis packs obtained from healthy volunteers (Etablissement Français du Sang). Adherent cells were removed by incubation on plastic dishes and passage over nylon wool columns. T lymphocytes were stimulated for 4 days with 2 μg/ml of the mitogenic GT2 + T11, CD2 mAb pair kindly given by Dr. A. Bernard (U 343, Nice, France) plus 100 units/ml interleukin-2 (IL-2). Primary discontinuous density Percoll gradients (Amersham Biosciences) were used to isolate large activated T cells as previously described (1, 22). These cells were exposed to 100–350 nM staurosporine (Sigma) for 90 min. Shrunken cells resulting from this treatment were isolated on a second density Percoll gradient. In some experiments, CD4+ and CD8+ T cells were separated prior to stimulation by immunomagnetic selection using anti-CD3 or anti-CD4-coated magnetic beads (Miltenyi Biotec, Auburn, CA). To evaluate changes in inner mitochondrial transmembrane potential Δψm, cells were stained for 15 min at 37 °C with 40 nM of the potential sensitive fluorescent dye DiOC6 (3,3′-dihexyloxacarbocyanine) from Molecube (Interchim, Montluçon, France). Cells with complete Δψm loss were obtained by a-10 min incubation with 5 μM m-CC1COP (carboxy cyanide m-chlorophenyl hydrazone, from Sigma). Condensed nuclei were examined after incubation of the cells for 5 min with 5 μM DAPI (Molecular Probes).

**Synthetic Inhibitors, Enzymatic Substrates, and Other Chemicals—**The pan-caspase inhibitor, BAF (Boc-Asp(OMe)-fluoromethylketone), and the Cat B and L inhibitor, Z-FA-fmk (benzoylxyloxy Carbonyl-Ph-ala fluoromethylketone), were from Enzyme Systems Products. The Cat B inhibitor, CA-074-Me, was from Calbiochem (France Biochem, Meudon, France). The Cat D inhibitor, pepstatin A, the Cat B substrate, Z-Arg-Arg-βNA (z-RR-βNA), the Cat L substrate, pepstatin A, the Cat B substrate, Z-Arg-Arg-g-βNA (z-RR-gNA), the Cat L substrate, hemoglobin, the β-hexosaminidase substrate, p-nitrophenyl N-acetyl-b-D-glucosaminide, and the inhibitor of vacuolar type H+-ATPase, bafilomycin A1, were from Sigma.

**Yeast Assay—**Cat B and Cat L were assayed by hydrolysis of their respective specific substrates z-RR-βNA and FR-βNA as described (24). Cat D activity was determined as described by Barret and Kirschke (24), with some modifications (25), by using hemoglobin as substrate. To measure Cat D activity in cytosolic fractions, cells were permeabilized with digitonin as described below. Hexosaminidase activity was assayed according to the protocol of Ref 26.

*Cathepsin D Triggers Bax Activation and AIF Release*

Among the cathepsins (Cat) and the use of small interfering RNA (siRNA) technique that has recently been used to study gene function in mammalian cells (22, 29), duplexes of 21-nucleotide siRNA with two 3′-overhanging TT were synthesized by Prologo (Paris, France). The sense strand of the siRNA used to silence the Cat D gene (Cat D-siRNA) GCUGGGCGGACCGUCAUAGAG (GUGGCUCGGGAACUGUCAUAGAG (GUGGCUCGGGAACUGUCAUAGAG), corresponding to position 684–688 related to the start codon of the Cat D mRNA). The sense strand of the siRNA silencing bax gene corresponded to the position 384–402 (GGUGCGGGAACUGUCAUAGAG) relative to start codon of the Bax-α mRNA (Bax-siRNA). The Bax-siRNA sequence is present in six differ-
ent Bax isoforms. The sense strand of the siRNA silencing aif gene (AIF-siRNA) was CUUGUUCCAGCGAUGGCAU (position 111–129 relative to start codon). Mock transfections were performed with buffer alone. An inefficient CD9 oligo (given by Dr. C. Boucheix, Inserm U268), corresponding to the GCCAUCCACUAUGCGUUGAAC sequence (position 334–354 relative to the start codon) was also used as a negative control of transfection (referred to as control-siRNA).

Transfection of purified resting T cells was carried out by electroporation using the Nucleofection® system (Amaxa, Köln, Germany), according to the protocols proposed by the furnisher. Briefly, 4 × 10⁶ T cells were resuspended in 100 μl of T cell nucleofector solution (Human T Cell Nucleofector kit) containing 75–150 pmol of double-stranded siRNAs. After electroporation, 400 μl of prewarmed cultured medium were added to the cuvette, and the cells were transferred into culture plates containing prewarmed culture medium. After a 16 h rest, T cells were activated as described above, and the expression of the targeted genes was monitored by Western blot at different times. At the optimal time of gene silencing (usually 96-h post-transfection), apoptosis was induced by STS.

**RESULTS**

Selective Mitochondrial Release of AIF during the Early Phase of Commitment to Apoptosis in Activated Primary T Lymphocytes—We have established an experimental system suitable for the isolation of a homogeneous population of activated T lymphocytes located in the early phase of commitment to apoptosis following a brief exposure to a low dose of STS (1). CD2- and IL-2-activated T cells, homogeneous in size, were first isolated as large cells in the low buoyant density fraction of discontinuous density Percoll gradients. These cells are re-
The percentages of cells with activated Bax was scored in CD4 +/H11001 incubated in 0.1M Na2CO3, pH 11.5, and centrifuged at 30 psi for 10 min to yield supernatant and pellet. The fractions were analyzed by Western blot for Bax and Cox II. In the mitochondrial pellets treated with Na2CO3, the increase in Cox II and Bcl-2 protein levels, occurring despite equivalent loading of protein (5 μg/lane), is likely due to the removal of loosely attached proteins, including Bax. Similar results were obtained in three other independent experiments.

FIG. 2. Bax acquires a membrane-inserting conformation in activated T lymphocytes that have entered the early commitment phase to apoptosis. A, confocal microscopy examination of the activation status of Bax, using an antibody raised against the 1–21 region of Bax (Bax-NT). Note the punctate Bax-NT immunostaining that colocalizes with the Hsp60 mitochondrial resident protein in STS-treated cells. B, Bax active conformation occurs in a caspase-independent manner. Activated T lymphocytes were pre-treated for 2h with 100 μM BAF and were then exposed to 100 nM STS for 90 min. Cells were stained with Bax-NT antibodies and positive cells were scored by conventional fluorescence microscopy (values are means ± S.D., n = 3). C, percentages of cells with activated Bax was scored in CD4 + and CD8 + T cells (n = 3). D, cytosolic and heavy membrane extracts were prepared from control and STS-treated cells. The fractions were analyzed by Western blot with anti-Bax, anti-Cox II, and anti-actin. Anti-cytochrome c was used to assess mitochondrial integrity. E, Bax translocated to mitochondria becomes alkali-resistant in STS-treated cells. The heavy membrane fraction from control and STS-treated cells was incubated in 0.1M Na2CO3, pH 11.5, and centrifuged at 30 psi for 10 min to yield supernatant and pellet. The fractions were analyzed by Western blotting for Bax and Cox II. In the mitochondrial pellets treated with Na2CO3, the increase in Cox II and Bcl-2 protein levels, occurring despite equivalent loading of protein (5 μg/lane), is likely due to the removal of loosely attached proteins, including Bax. Similar results were obtained in three other independent experiments.

FIG. 3. Bax translocation to mitochondria is not associated with proteolytic activation of Bid. A, Bid remains as a full-length protein in the cytosol of activated T lymphocytes treated with 100 nM STS. Bax, Bid, Cox II, and actin were analyzed by Western blot in cytosolic and heavy membrane extracts. B, immunoblot showing that full-length Bid translocates to the mitochondria at a higher STS concentration (350 nM). The data are representative of four independent experiments.

The results were obtained in three other independent experiments. Following exposure to 100 nM STS for 90 min, most of these cells (82 ± 7%, n = 5) displayed an increase in density and a decrease in volume (Fig. 1A, panel a). This made it possible to recover the shrunken cells on a second Percoll density gradient. As previously shown (1), most of these cells maintained a high ΔΨm (85–90% DIOC6 high cells) but displayed peripheral chromatid condensation (panel b). They also displayed diffuse immunostaining of AIF, which spread into the cytoplasm and the nucleus, contrasting with the punctate immunostaining of cytochrome c seen on confocal (panel c) or conventional (panel d) microscopy. These changes occurred in the presence of BAF, a cell-permeable pan-caspase inhibitor, corroborating our previous observation that caspases are not involved at this stage (1).

Like AIF and cytochrome c, the apoptogenic proteins Smac/DIABLO, Omi/HtrA2, and endonuclease G (EndoG) reside in the intermembrane space of mitochondria, awaiting influx in response to apoptotic stimuli (reviewed in Ref. 30, see also “Discussion”). Subcellular fractionation of the cells into P10 heavy membrane pellets (enriched in mitochondria) and cytosolic S100 fractions, followed by immunoblotting (Fig. 1, B and C), indicated that STS readily provoked the partial release of AIF from mitochondria into the cytosol but did not significantly induce the release of other apoptogenic factors from the mitochondria (Fig. 1B, lane b). In T lymphocytes at more advanced stages of apoptosis (lane c, exposure to 350 nM STS for 90 min, resulting in >30% ΔΨm low cells), mitochondrial apoptogenic factors displayed a higher level of release into the cytosol (AIF and Omi/HtrA2) or even complete redistribution (cytochrome c, Smac/DIABLO, and EndoG). Thus, AIF was selectively translocated to the cytosol during the early phase of commitment to apoptosis in our system.

Bid-independent Activation of Bax—Untreated and STS-treated cells were stained with rabbit antibodies directed against amino acids 1–21 of Bax (Bax-NT, green fluorescence) and examined by confocal microscopy, using an anti-Hsp60 antibody (red fluorescence) as a mitochondrial marker. No green fluorescence was detected in control cells whereas, after 90 min of STS treatment, such fluorescence was clearly visible and was almost completely co-localized with Hsp60-associated fluorescence (yellow staining, Fig. 2A). Conventional immunofluorescence microscopy showed that at least ≥70% of STS-treated T cells exhibited the Bax N terminus, irrespective of whether BAF was present. Bax conformational change occurred in both CD4 + and CD8 + T cells, to similar extents (Fig. 2, B and C). We further investigated the subcellular distribu-
Somatic destabilization. Apoptosis is correlated with limited lysosomal destabilization. A, the pictures show the punctate staining for all cathepsins in control cells and the mixed diffuse/punctate staining in STS-treated cells, assessing partial lysosomal release (fluorescence microscopy). B, LysoTracker-red uptake by activated T cells; the granular staining in STS-treated cells is consistent with a normal cytoplasmic-lysosomal pH gradient. Cells exposed to 100 nM Baf A1 for 1 h were not stained. C, cells were incubated for 2 h with 5 mg/ml FITC-dextrans of different molecular weights. After a 2-h chase period, they were exposed to STS for 90 min. White numbers within the pictures represent the percentages of cells exhibiting a mixed granular/diffuse staining. Inserted pictures correspond to cells doubly stained with anti-Cat B (red) and FITC-dextran, showing entire co-localization. D, β-hexosaminidase activity was measured in cytosolic fractions obtained by digitonin-based plasma membrane permeabilization.

**Fig. 4.** Lysosomal efflux of Cat B, D, and L in T lymphocytes committed to apoptosis is correlated with limited lysosomal destabilization. A, the pictures show the punctate staining for all cathepsins in control cells and the mixed diffuse/punctate staining in STS-treated cells, assessing partial lysosomal release (fluorescence microscopy). B, LysoTracker-red uptake by activated T cells; the granular staining in STS-treated cells is consistent with a normal cytoplasmic-lysosomal pH gradient. Cells exposed to 100 nM Baf A1 for 1 h were not stained. C, cells were incubated for 2 h with 5 mg/ml FITC-dextrans of different molecular weights. After a 2-h chase period, they were exposed to STS for 90 min. White numbers within the pictures represent the percentages of cells exhibiting a mixed granular/diffuse staining. Inserted pictures correspond to cells doubly stained with anti-Cat D (red) and FITC-dextran, showing entire co-localization. D, β-hexosaminidase activity was measured in cytosolic fractions obtained by digitonin-based plasma membrane permeabilization.

**Cytosol upon STS Apoptotic Signaling**—Lysosomal cathepsins have been shown to be readily translocated to the cytosol and to mediate apoptosis in response to various stimuli (reviewed in Ref. 20). We therefore investigated the subcellular distribution of Cat B and L, both cysteine proteases, and of Cat D, an aspartic protease, all three of which are abundant in lysosomes. In control activated T cells, immunostaining of cathepsins B, D, and L displayed a punctate distribution on immunofluorescence microscopy, consistent with a lysosomal location. In contrast, in cells treated with STS for 90 min, these cathepsins displayed a mixed punctate/diffuse distribution, indicating that only part of them was translocated to the cytosol (Fig. 4A, see also the Western blot of Fig. 7A). No cathepsin was found in the nucleus, as assessed by confocal microscopy (not shown). The cytosol-lysosome pH gradient seemed to be preserved in STS-treated cells because LysoTracker-Red, an acidic organelle-specific probe, still accumulated in granular cellular structures. In contrast, LysoTracker-Red uptake was completely prevented in cells preincubated with 100 nM bafilomycin A1 (Baf A1), a specific inhibitor of the vacuolar H+-ATPase that abolishes acidification in lysosomes (31) (Fig. 4B). To evaluate the extent of lysosomal permeabilization, the cells were preloaded with FITC-conjugated dextrans of different molecular weights, and after a 2-h chase (allowing complete co-localization with Cat D), exposed to STS (Fig. 4C). In these conditions, the 10-kDa FITC-dextran molecules were relocated to the cytosol in the majority of cells, while the redistribution of the 40-kDa FITC-dextran molecules was observed in only 44% of the cells. In both cases, the molecules displayed a mixed diffuse/punctate distribution, indicative of partial lysosomal efflux. In contrast, the larger 70 and 250-kDa dextrans remained in the lysosomes. We also tested β-hexosaminidase activity in the cytosolic fractions obtained by digitonin-based plasma membrane permeabilization, and observed that this 250-kDa enzyme, which is normally resident in the lysosomes, was almost totally retained within these organelles (Fig. 4D). Thus, the release of proteins from lysosomal vesicles was rather size selective. Collectively, these data suggest that limited, but no large scale lysosomal destabilization was occurring in activated T cells committed to apoptosis, allowing the nonspecific release of cathepsins B, D, and L molecules (and possibly other lysosomal constituents), all of which display a molecular mass below 70 kDa.

**The Inhibition of Cat D Activity by Pepstatin A Prevents Bax Activation and AIF Release**—To test the specific role of cathepsins, inhibitors of Cat D (pepstatin A), Cat B (CA074-Me), and Cat B and L (Z-FA-dmk), were added to the cells 2 h before exposure to STS. The specificity of the Cat inhibitors in our system was confirmed by assaying cathepsins for hydrolysis of...
same doses of Cat inhibitors and then exposed to 100 nM STS for 90 min. B, kinetics of STS-induced cell death in the presence or absence of pepstatin A. Percentages of activated T cells remaining viable (excluding trypan blue) after STS treatment are related to the percentages of viable cells in untreated control T cells (arbitrary value 100%). Values are means ± S.D. of three independent experiments.

dria (Fig. 5C). Nor was it inserted into the OMM (Fig. 5D). These data indicate that the lysosomal efflux of Cat D occurred upstream from the change in conformation of Bax, its relocation, and membrane insertion. Lysosomal efflux of Cat D also occurred upstream from mitochondrial AIF release.

Ultrastructural examination of the lymphocytes showed that their heterochromatin, which was condensed in discrete clumps following STS exposure, had a normal aspect in pepstatin A-treated cells. There was no longer volume loss (Fig. 6A). Thus, the pretreatment of activated T lymphocytes with pepstatin A abolished the early apoptotic phenotype induced by STS. In the kinetics illustrated in Fig. 6B, it is shown that 50% of the cells exposed to STS died after 2 h and ~80% after 8 h. Pepstatin A completely halted apoptosis for ~5 h, but no longer, suggesting that death triggers other than Cat D came into play at this time. Thus, Cat D is the sole trigger of apoptosis for the first 5 h after exposure to the death signal.

Cathepsin D Is Stable at Neutral pH and Can Still Trigger, in the Presence of Bafilomycin A₁, the Apoptotic Events of the Early Commitment Phase to Apoptosis—Western blot analysis indicated that the mature (32 kDa) form of Cat D was present in the cytosolic fractions obtained from STS-treated cells, but not from control cells, after permeabilization of the plasma membranes with digitonin (Fig. 7A, panel a). Part only of lysosomal Cat D was released into the cytosol as compared with the amount of Cat D recovered after solubilization of cell membranes with Triton X-100. Released Cat D was catalytically active in a standard hydrolysis assay performed at pH 3.5, using hemoglobin as a substrate (Fig. 7A, panel b). To examine whether the inhibition of lysosomal acidification would affect the early apoptogenic events, control activated T cells were first preincubated with Baf A₁. Fig. 7B shows that after 1 h exposure to 25–100 nM Baf A₁, the uptake of LysoTracker-Red was almost totally abolished. However, Cat D remained fully oper-
ative when assayed for activity at pH 3.5. The cells were in all cases healthy, displaying intact plasma membranes (not shown). Baf A1 did not prevent, in STS-treated cells, the release of CatD in the cytosol, the appearance of the active form of Bax (Bax-NT), the mitochondrial efflux of AIF, the appearance of condensed nuclei (Fig. 7C, panel a), and the loss in cell volume (Fig. 7C, panel b). That Cat D was catalytically active in this setting was demonstrated by the inhibitory effect exerted by pepstatin A on these apoptotic events. Thus, inhibition of the vesicular H^+ -ATPase by Baf A1 had no influence on Cat D-induced early commitment to apoptosis.

siRNA-induced Silencing of Cat D and Bax Genes Allows the Definition of a Cat D-initiated Pathway Leading to Mitochondrial Perturbation—In addition to its downstream effect on Bax activation, Cat D may also have provoked mitochondrial membrane perturbation by means of an alternative pathway. Recent reports have demonstrated the efficacy of gene silencing by siRNA in mammalian cells (29) including primary T cells (32). We therefore used the siRNA-mediated gene silencing technique to examine the sequential relationship between Cat D activity, Bax conformational activation, and mitochondrial AIF release. Resting T cells were first transfected with siRNAs targeting the human Cat D mRNA. Optimal transfection conditions were 1.5 μM Cat D-siRNA/4 x 10^6 cells. siRNA-mediated gene silencing became fully operative 4 days post-transfection. These conditions led to Cat D protein production being strongly decreased in activated T lymphocytes after a 5-day CD2 stimulation period, whereas Cat B protein production was not affected, as revealed by immunoblotting and immunofluorescence (Fig. 8A). Mock-transfected cells, and cells transfected with control-siRNA had normal levels of Cat D protein. All transfected cells were healthy (90% DiOC6 high), and reacted to the mitogenic stimulus in the same way as mock- or control-siRNA-transfected cells in terms of cell number and size (not shown). Following exposure to STS for 90 min, these cells no longer displayed Bax-NT immunoreactivity (Fig. 8B) nor AIF relocation (Fig. 8C). They did not undergo cell shrinkage (see FSC values in Fig. 8B, panel a). We then investigated the effects of siRNA-mediated Bax gene silencing. Transfection with 0.75 μM Bax-siRNA optimally reduced Bax protein levels but did not affect Bid protein levels, as shown by Western blotting (Fig. 9A). Consistent with this, Bax-associated immunofluorescence was barely detected in the cells, contrasting with the bright, punctate Cat D-associated immunofluorescence (Fig. 9B). In these conditions, STS apoptotic signaling induced the redistribution of Cat D throughout the cytosol. In contrast, AIF gave a punctate pattern of staining, indicating that it was retained in the mitochondria. Thus, Bax activation is a selective event occurring downstream from Cat D activity and resulting in permeabilization of the OMM and AIF release.

**Fig. 7.** Bafilomycin A1 does not prevent lysosomal Cat D efflux nor the occurrence of apoptotic events in STS-treated lymphocytes. A, cytosolic extracts of control and STS-treated cells obtained by the digitonin-based plasma membrane permeabilization technique. Panel a, immunoblot analysis using anti-Cat D. Anti-cytochrome c was used to verify the integrity of the mitochondria, and anti-actin to assess an equal protein loading. The total content of lysosomal Cat D was obtained by treating control cells with 1% Triton X-100 instead of digitonin. Panel b, the activity of Cat D was evaluated in the cytosolic extracts by using hemoglobin as a substrate at pH 3.5. B, Cat D proteolytic activity against hemoglobin at pH 3.5 and LysoTracker-Red uptake in the lysates of cells pretreated for 1 h with increasing concentrations of Baf A1. C, Cat D proteolytic activity in cells pretreated for 1 h with both 100 nm Baf A1 and 25 μM pepstatin A. C, cells pretreated with 100 nm Baf A1, and 25 μM pepstatin A before being subjected to 100 nM STS for 90 min. Panel a, percentages of cells with diffuse Cat D, active Bax, diffuse AIF, and condensed nuclei (means ± S.D., n = 3). Panel b, decrease in cell size analyzed by FSC.
Fig. 8. Depletion of Cat D protein by RNA interference results in the suppression of Bax activation and of AIF release in STS-treated cells. A, resting T lymphocytes were transfected with Cat D-siRNA. Negative controls consisted of untransfected cells, of mock-transfected cells, and of cells transfected with an inefficient siRNA (control-siRNA). 16 h later, the cells were stimulated with the mitogenic CD2 mAb pair (GT2 + T11.1) for 4 days. Western blot analysis of whole cell lysates in panel a shows that the Cat D protein expression is inhibited in cells transfected with 1.5 μM of Cat D-siRNA, whereas, for the same protein loading (see actin), the level of Cat B protein is unaffected. Panel b, extinction of Cat D-associated immunofluorescence in Cat D-siRNA treated cells (confocal microscopy). Panel c, percentages of cells displaying Cat D-associated immunofluorescence (cells are detected by means of their DAPI-positive nuclei in conventional fluorescence microscopy). B, 4 days after CD2 mitogenic stimulation, Cat D-siRNA-transfected T lymphocytes were incubated with 100 nM STS for 90 min. The cells were then simultaneously stained with anti-Bax-NT and anti-ti-Hsp60, examined by confocal microscopy (panel a) and counted for Bax-NT associated immunofluorescence by conventional microscopy (panel b). Values in white inside the pictures of panel a are means FSC, indicating that the cells transfected with Cat D-siRNA do not undergo shrinkage. C, AIF subcellular localization in siRNA-transfected T lymphocytes exposed to STS. Panel a, in control-siRNA-transfected cells, AIF displays a mitochondrial punctate distribution pattern, and is relocated (diffuse staining) after STS treatment. In Cat D-siRNA transfected cells, the distribution pattern of AIF protein remains punctiform after STS treatment; panel b, percentages of cells with diffuse AIF. All histograms are means ± S.D. obtained by counting 300 cells from triplicate cultures. The experiment depicted in the figure is representative of triplicates.

We can also conclude that Cat D does not directly perturb mitochondria. In kinetics studies (data not shown), we estimated that the percentage of cells displaying diffuse (relocated) Cat D staining reached a plateau 10 min after the apoptosis trigger. The plateau for Bax-NT staining was reached after 20 min, and that for diffuse AIF staining was reached last of all, after 30 min. This pattern of staining provides a precise picture of the temporal relationship between these molecular events.

Down-regulation of AIF Protein Production by AIF-siRNA Abolishes the Early Apoptotic Phenotype and Delays Cell Death—T cells transfected with AIF-siRNA (1.5 μM), and examined by Western blot 4 days after mitogenic stimulation, displayed a substantial decrease in AIF protein levels (60% decrease as estimated by densitometric analysis), whereas cytochrome c protein levels remained stable (Fig. 10A). Although some AIF protein was always present, the number of AIF-positive cells in immunofluorescence was decreased by ≈72%. In these conditions, STS induced full Cat D relocalization and Bax activation (Fig. 10B). However, cell shrinkage was prevented, as seen by forward scatter analysis, and the number of cells with condensed nuclei was decreased, as visualized by DAPI staining (Fig. 10C). Importantly, cell death was halted for a few hours, assessing that AIF controls early cell death, downstream from Cat D activity and Bax activation.

DISCUSSION

The aim of this study was to investigate the proapoptotic factors mediating the mitochondrial destabilization that occurs during the early caspase-independent phase of commitment to apoptosis in activated T lymphocytes. We used STS as a cell death inducer because it mimics apoptotic stresses that stimulate the intrinsic mitochondrial death pathway in which Bax and Bak are obligatory but redundant executioners (33). In activated T lymphocytes, the apoptotic pathway induced by STS at low dose may be related to "death-by-neglect," a process which takes place after antigen clearance and inflammatory cytokines withdrawal, and which is believed to eliminate excess numbers of activated T lymphocytes at the end of an immune response (33). Using peptide inhibitors specific for Cat B, D, and L, and by introducing siRNAs complementary to Cat D and Bax mRNAs into T lymphocytes, we demonstrated that Cat D, an aspartic protease released from the lysosomes into the cytosol, is an upstream trigger of Bax activation. Bax in turn, provokes limited OMM destabilization, independently of Bid, resulting in selective AIF release. We also demonstrated that down-regulation of AIF protein levels by siRNA complementary to AIF mRNA prevents the early apoptotic events induced by STS, such as cell shrinkage and nucleus condensation. The inhibition of Cat D by pepstatin A or the decrease in...
AIF protein levels delayed the appearance of the apoptotic phenotype and prevented cell death for several hours, but apoptosis was then reinitiated, indicating that Cat D effect was then superseded by other dominant apoptotic mechanisms at this time.

Cat D has been shown to act as a death inducing factor in several apoptosis systems (25, 34–36). It is proteolytically active at the acidic pH of the lysosomes and endosomes. Data from the literature however support the notion that extralysosomal Cat D may be active at the neutral pH of the cytosol. Thus, microinjection of Cat D causes apoptosis (37) and limited disruption of lysosomes by lysosomotropic reagents triggers an apoptosis-like cell death largely induced by Cat D (38). In our experiments, Cat D was present in the cytosolic fractions of STS-treated cells as a mature p32 enzyme that could degrade hemoglobin in a standard hydrolysis assay at pH 3.5. Baf A1, a vacuolar-type H^+/-ATPase that inhibits lysosomal acidification, not only failed to prevent the lysosomal efflux of cathepsins, but also failed to inhibit the early apoptotic events of the commitment phase. These events were however prevented by pepstatin A, indicating that Cat D was still active in Baf A1-treated cells. If Cat D stability and activity resist Baf A1, one may conceive that released Cat D can interact with target cytosolic proteins at neutral pH. Using crystal structures of Cat D, Lee et al. (39) have shown that the occupancy of the active site by pepstatin A stabilizes the active conformation of Cat D at neutral pH. This suggests that substrates with the highest affinity will probably do the same. In this view, released Cat D might activate Bax by exerting its proteolytic activity on an intermediary cytosolic molecule. In our study, Bid remained as an inactive full-length p21 protein in the cytosol, and thus could not facilitate Bax conformational change. Bid lacks the target sequence recognized by Cat D (40), and pepstatin A, an inhibitor of Cat D, does not inhibit Bid proteolysis in cells harboring...
photodamaged lysosomes (41). Released Cat D might degrade a cytosolic chaperone that sequesters Bax in an inactive conformation in healthy cells. Possible candidates for this chaperone include certain isoforms of the cytosolic 14-3-3 proteins that interact directly with Bax and undergo dissociation during apoptosis (42), and the Ku70 protein that binds to the N terminus of Bax, preventing its translocation to mitochondria (43).

Cat D was translocated to the cytosol together with Cat B and Cat L, but the lysosomal efflux of these cathepsins was only partial. If the cells were loaded with FITC-dextran molecules of increasing molecular weights, only the molecules below 70 kDa were found to be released upon exposure to STS. These data argue for a mechanism of limited destabilization of the lysosomes resulting in the partial release of cathepsins, and probably other lysosomal constituents, in a size selective manner. The mechanism(s) accounting for lysosomal permeabilization are still elusive. Whatever, only Cat D triggered the apoptotic events occurring during the early and narrow time window of our observations. Cat B can also act as a death factor (28, 44), but the extralysosomal proteolytic activity of Cat B can be prevented by cytosolic “emergency inhibitors” known as steffins, which inhibit cysteine proteases (reviewed in Ref. 45).

No such inhibitor is known for the aspartic protease Cat D, possibly accounting for the predominant role of this enzyme at the onset of lysosomal destabilization.

The mitochondrial perturbation caused by activated Bax during early commitment to apoptosis was characterized by the selective escape of AIF from mitochondria. AIF release was caspase-independent, consistent with the results of Susin et al. (3) but contrasting with recent findings showing that the mitochondrial release of AIF is inhibited by caspase inhibitors (46). In C. elegans, the translocation of the AIF homolog, WAH-1, is mainly dependent on the caspase homolog CED-3 (8). AIF has been shown to be attached to the inner mitochondrial membrane rather than as a soluble intermembrane space protein (46). There may be two waves of mitochondrial release of AIF from the mitochondria. The first wave (seen in our system) would be rapid, caspase-independent, and would affect only partial AIF molecules, sparing those attached to the inner membrane (see Fig. 1). The second wave, which would be caspase-dependent, would further deplete the remaining mitochondrial AIF pool. AIF is probably assimilated in vivo by EndoG to promote efficient DNA degradation (8). Peripheral chromatid condensation occurs without large scale and oligosomal DNA fragmentation in the cells of our system (1), in line with the fact that EndoG remains in the mitochondria. Our study shows in addition that down-regulation of AIF protein production by siRNA-mediated gene silencing abolishes the early apoptotic phenotype, indicating that AIF alone is sufficient to induce its occurrence.

Mice deficient in Cat D develop normally during embryogenesis and the first 2 weeks but die around day 25, exhibiting atrophy of the intestinal mucosa and massive destruction of T and B cells (47). The harlequin (Hq) mutant mice, which display a profound decrease in AIF mRNA and protein levels, have cerebellar and retinal neurons particularly sensitive to cell death induced by oxidative stress (48). In our experiments, Cat D- and AIF-siRNA transfected T lymphocytes were as healthy as untransfected control cells. Thus, the time course of our study was short enough to avoid the deleterious effects of Cat D or AIF deficiency.

In conclusion, by inactivating expression of the Cat D, Bax, and AIF genes by RNA interference, and using a series of 4-2 cathepsin inhibitors, we have demonstrated a novel apoptotic pathway by which Cat D induces mitochondrial destabilization and AIF release via Bax, and by which translocated AIF controls the early apoptotic phenotype in activated T lymphocytes.

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