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Active Caspase-3 Is Stored within Secretory Compartments of Viable Mast Cells

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Caspase-3 is a main executioner of apoptotic cell death. The general notion is that, in viable cells, caspase-3 is found as a cytosolic inactive proenzyme and that caspase-3 activation is largely confined to processes associated with cell death. In this study, we challenge this notion by showing that enzymatically active caspase-3 is stored in viable mast cells. The enzymatically active caspase-3 was undetectable in the cytosol of viable cells, but was recovered in subcellular fractions containing secretory granule-localized proteases. Moreover, active caspase-3 was rapidly released into the cytosolic compartment after permeabilization of the secretory granules. Using a cell-permeable substrate for caspase-3, the presence of active caspase-3–like activity in granule-like compartments close to the plasma membrane was demonstrated. Moreover, it was shown that mast cell activation caused release of the caspase-3 to the cell exterior. During the course of mast cell differentiation from bone marrow cells, procaspase-3 was present in cells of all stages of maturation. In contrast, active caspase-3 was undetectable in bone marrow precursor cells, but increased progressively during the process of mast cell maturation, its accumulation coinciding with that of a mast cell–specific secretory granule marker, mouse mast cell protease 6. Together, the current study suggests that active caspase-3 can be stored within secretory compartments of viable mast cells. The Journal of Immunology, 2013, 191: 1445–1452.

Mast cells are main effector cells of the immune system, being implicated in a variety of pathological contexts, including allergic disorders, cancer, atherosclerosis, arthritis, and host defense (1). A hallmark characteristic of mast cells is their large content of lysosome-like secretory granules, filled with numerous preformed bioactive compounds such as histamine, serotonin, cytokines, serglycin proteoglycans, lysosomal enzymes, and different mast cell–specific proteases, the latter comprising tryptases, chymases, and carboxypeptidase A3 (CPA3) (2). When mast cells are activated, a process that can be accomplished in many ways, the cells degranulate and thereby release the contents of their secretory granules to the cell exterior (3).

The caspases comprise a family of ubiquitously expressed cysteine proteases (4). The caspase family can be subdivided into the proinflammatory members (caspase-1, -4, -5, -12) and into caspases lacking an endoplasmic reticulum localization signal peptide, that is, it is translated in the cytosol and is localized in this compartment as an inactive proenzyme under latent conditions. Upon the induction of apoptosis, procaspase-3 is proteolytically activated, and activated caspase-3 then executes many of the hallmark events of apoptosis (4, 8). Hence, procaspase-3 activation is generally considered as an event associated with apoptotic cell death. Consequently, procaspase-3 is the predominant form present in living cells, whereas active caspase-3 is normally not found. In this study, we present evidence that contests this general notion, by showing that enzymatically active caspase-3 is stored within granule-like compartments of live mast cells and that mast cells can release enzymatically active caspase-3. Moreover, we show that storage of activated caspase-3 accompanies the differentiation of precursor cells into mature mast cells.

Materials and Methods

Reagents

H-Leu-Leu-OMe-HBr (t-leucyl-t-leucine methyl ester [LLME]) was from Bachem (Bubendorf, Switzerland). Digitonin was from Sigma-Aldrich (Steinheim, Germany). Rabbit anti–procaspase-3 and anti-activated caspase-3 (G.G.-F. and F.R.M. contributed equally to this work.

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; CPA3, carboxypeptidase A3; LDH, lactate dehydrogenase; LLME, t-leucyl-t-leucine methyl ester; mMC6, mouse mast cell protease 6; PCMC, peritoneal cell–derived mast cell; SCF, stem cell factor.

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mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA [pH 7.4]) and stimulated with TNF-α (0.4 μg/ml). Alternatively, 1 μM A23187 was used. At different time points of incubation, media and cell fractions were frozen and stored at −20°C until analysis. Control experiments showed that A23187 was not toxic for the mast cell populations studied (data not shown), β-Hexosaminidase activity was assayed, as described (9).

Preparation of granule-enriched fractions
Granule-enriched fractions were obtained from BMMCs by soft disruption in a bath sonicator and density centrifugation in Percoll, as previously described (10).

Enzymatic assays
Caspase-3–like activity was measured using the Z-DEVD-R110–based EnzChek caspase-3 assay kit 2 procedure (Molecular Probes/Invitrogen, Carlsbad, CA), and fluorescence intensity was monitored at 496 nm (excitation) and 520 nm (emission). Cysteine cathepsin-like activity was measured using 20 μM Z-Phe-Arg-AMC (Bachem). AMC release was monitored at 390 nm (excitation) and 460 nm (emission). Lactate dehydrogenase (LDH) was determined by a decrease in absorbance at 340 nm, resulting from the oxidation of 0.2 mM NADH. Trypsin-like activity was measured, as previously described (11). Histone deacetylase activity was measured using the HDAC Assay Kit, Fluorometric Detection (Millipore, Billerica, MA), following instructions from the manufacturer. For all measurements, samples were transferred in duplicates into individual wells of 96-well flat-bottom plates, and readings were performed at room temperature using a microplate reader (Infinite M200; Tecan).

Immunoblot analysis
Samples corresponding to equal numbers of cells were solubilized in SDS-PAGE sample buffer containing 5% DTT, as previously described (12). Membranes were scanned using an Odyssey Infrared Imager (Li-cor).

Digitonin extracts and cytosolic extract preparation
BMMCs (10⁶ cells) were collected by centrifugation (1200 rpm, 8 min, 4°C) in 1.5-ml Eppendorf tubes and then resuspended in 300 μl different concentrations of digitonin in ice-cold digitonin extraction buffer (250 mM sucrose, 20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA). After 10-min incubation on ice, the supernatant was quickly removed by centrifugation (13,000 rpm; 2 min, 4°C) and frozen (−20°C). Cytosolic extracts were obtained at the same conditions by using 10 μg/ml digitonin in ice-cold digitonin extraction buffer.

Caspase-3–like activity in live cells
Aliquots of 100 μl from 0.5 × 10⁶ cells/ml suspension were transferred into individual wells of a 96-well flat-bottom plate, in the presence and/or absence of 20 μM Z-DEVD-FMK (Sigma-Aldrich, Steinheim, Germany). After 1-h incubation at 37°C in 5% CO₂, 5 μM PhiPhLux-G-Q₂ (Oncomimunin, Gaithersburg, MD) was added, followed by incubation for 2 h. Thirty and 10 min before the end of the incubation time, 20 μM calcein-AM (Invitrogen, Carlsbad, CA) and 0.3 μg DAPI were added. Cells were then transferred into 1.5-ml tubes and washed twice with 300 μl PBS. Pellets were resuspended in 100 μl PBS, transferred to microscopy glasses, fixed with 4% paraformaldehyde for 15 min, and covered with fluoromount-G mounting medium from Southern Biotech, Birmingham, AL and cover glass. The entire assay was performed in the dark. Samples were analyzed using a laser-scanning microscope equipped with ZEN 2009 software (LSM 710; Carl Zeiss, Berlin, Germany).

Total RNA isolation and quantitative PCR
Total RNA was isolated and analyzed for levels of caspase-3 mRNA, as described (9), using the following primers for caspase-3: 5′-TCAG-AOGCGACTCTCCTCGGAGA-3′ as forward primer and 5′-CCACCCTGTATCCCTTGGCGAAGCC-3′ as reverse primer. Hypoxanthine guanine phosphoribosyl transferase was used as housekeeping gene using the primers specified previously (9).

Statistical analysis
Data shown are means ± SEM. Statistical analyses were performed by using GraphPad Prism 4.0c (GraphPad Software) and paired Student t test for two-tailed distributions (*p < 0.05, **p < 0.01, ***p < 0.001).

Results
Active caspase-3 is present in mast cells and is released by mast cell activation
In a previous study, we showed that mast cells are sensitive to cell death induced by secretory granule-permeabilizing agents, and that mast cell apoptosis in response to such agents is accompanied by caspase-3 activation (13). Unexpectedly, we noted signs of the presence of active caspase-3 even in the absence of cell death-inducing agent (13). Possibly, the activated caspase-3 could have been present in an apoptotic subpopulation of the primary mast cell population used for those studies. However, we in this study decided to investigate the alternative possibility, that is, that enzymatically active caspase-3 may in fact be present in viable mast cells. For this purpose, we developed mast cells by differentiation of bone marrow cells into mature mast cells, that is, BMMCs (9). In addition, we derived mast cells by expansion of the peritoneal mast cell population, that is, peritoneal cell–derived mast cells (PCMCs) (14).

To assay for caspase-3 activity, we used Z-DEVD-R110. As shown in Fig. 1A, BMMCs indeed possessed caspase-3–like activity in the absence of cytotoxic agent, and this activity was completely abrogated by addition of a caspase-3 inhibitor (Ac-DEVD-CHO). Moreover, immunoblot analysis using an Ab detecting active caspase-3 (but not the proenzyme) revealed the presence of processed caspase-3 in BMMCs (Fig. 1B). Notably, two bands were recognized by the anti–caspase-3 Ab, migrating at positions corresponding to ~22.5 and ~19.5 kDa. These bands are thus in agreement with the partly processed, enzymatically active caspase-3 forms recently reported by Pederzoli et al. (15). Both of these bands were confirmed to correspond to caspase-3, as judged by abrogated signals in the presence of a blocking peptide (Fig. 1B).

Because active caspase-3 is unlikely to be present in the cytosol of viable cells, we considered the possibility that it may be sequestered within secretory granules. To address this possibility, we added agents that cause mast cell degranulation and assessed whether this result in release of active caspase-3. As seen in Fig. 1C, release of caspase-3–like activity into the medium was indeed induced by the calcium ionophore A23187, an agent that is known to produce robust mast cell degranulation. However, caspase-3 activity was also found in the cell supernatant in the absence of mast cell–activating stimuli, suggesting that active caspase-3 is released spontaneously by mast cells. In the presence of a caspase-3 inhibitor (Ac-DEVD-CHO), the secreted caspase-3–like activity was almost completely abrogated (Fig. 1C), whereas a general inhibitor of serine proteases (Pefabloc SC) had no effect. The addition of calcium ionophore did not cause any increase in the levels of caspase-3 mRNA, indicating that the increase in extracellular caspase-3 activity was not a consequence of increased caspase-3 mRNA expression (Fig. 1D). Moreover, the release of caspase-3 activity into the medium was accompanied by a decrease in the contents of intracellular caspase-3, as judged by both activity measurements (Fig. 1A) and immunoblot analysis (Fig. 1B). In contrast to the response toward calcium ionophore, we did not detect any increase in caspase-3 release in response to IgE receptor cross-linking (Fig. 1A). As a positive control to verify that the used stimulants induce mast cell degranulation, the stimulation by both calcium ionophore and IgE receptor cross-linking was shown to cause release of β-hexosaminidase, a granular marker (Fig. 1E).

To extend these findings, we assessed whether enzymatically active caspase-3 was also found in PCMCs, a cell population that represents terminally differentiated mast cells (14). Indeed, processed caspase-3 was detected by immunoblot analysis of cell extracts from PCMCs (Fig. 2A). Moreover, activation of PCMCs
with calcium ionophore resulted in increased release of caspase-3 activity into the medium (Fig. 2B), accompanied by a depletion of cellular caspase-3 (Fig. 2A). In contrast, IgE receptor cross-linking did not cause a significant depletion of cell-associated caspase-3 (Fig. 2A) or any significant increase in the release of caspase-3 into the medium (Fig. 2B). Activation of the PCMCs by calcium ionophore or by IgE receptor cross-linking did not cause any significant effects on caspase-3 mRNA expression (Fig. 2C). Approximately 25 and 8% of total caspase-3–like activity was released by BMMCs and PCMCs, respectively, in response to calcium ionophore stimulation, whereas ~60% of the total trypsin activity was released.

**Accumulation of active caspase-3 accompanies the differentiation of bone marrow precursor cells into mature mast cells**

To investigate whether the presence of active caspase-3 is linked to mast cell phenotype, we studied the expression of caspase-3 during the course of differentiation of bone marrow precursor cells into mature BMMCs. As expected, based on the ubiquitous expression of caspase-3, caspase-3 mRNA was found in cells of all stages of maturation (Fig. 3A). Notably though, the level of caspase-3 mRNA increased progressively as the cells matured into mature mast cell phenotype. An increase in caspase-3 mRNA expression in maturing mast cells was apparent using three different protocols for inducing mast cell differentiation, each producing a mast cell population of distinct phenotype (9, 16, 17). To produce mast cells with a phenotype that resembles that of connective tissue-type mast cells, cells were cultured in either WEHI-3B–conditioned medium (contains IL-3) or IL-3 plus stem cell factor (SCF); produces BMMCs of more mature connective tissue-type mast cell–like phenotype. To produce mast cells with a phenotype that resembles that of mucosal-type mast cells, cells were cultured in IL-3 plus SCF plus IL-9 plus TGF-β (Fig. 3A).

At the protein level, procaspase-3 was found in approximately equal amounts in cells of all stages of maturation (Fig. 3B, upper panel). In contrast, active caspase-3 was undetectable in the bone marrow cell population (day 0) and at early stages of mast cell maturation, but was clearly detected in cells of later stages of mast cell maturation (Fig. 3B, middle panel). At all stages of cell culture, cell viability exceeded 95%, with no increase in the percentage of dead cells in late-stage cultures. Hence, the accumulation of caspase-3 in late-stage cultures is not a reflection of an increased population of nonviable, apoptotic cells. Notably, the accumulation of active caspase-3 coincided with the accumulation of mouse mast cell protease 6 (mMCP-6), the latter being a mast cell–specific protease localized to the secretory granules (18) (Fig. 3B, lower panel). The levels of caspase-3 protein were similar in mature mast cell populations of distinct phenotype (9, 16, 17). To produce mast cells with a phenotype that resembles that of connective tissue-type mast cells, cells were cultured in IL-3 plus SCF plus IL-9 plus TGF-β (Fig. 3A).

**FIGURE 1.** Active caspase-3 is present in mast cells and is released by mast cell degranulation. BMMCs (4 × 10⁶ cells) were either nontreated (control) or induced to degranulate using calcium ionophore (A23187) or IgE receptor cross-linking (IgE-TNP/OVA). (A) After centrifugation, cell pellets were solubilized and cell extracts were analyzed for caspase-3–like activity, in the absence or presence of either a caspase-3 inhibitor (Ac-DEVD-CHO) or a serine protease inhibitor (Pefabloc SC), as indicated. (B) Immunoblot analysis for active caspase-3 using an Ab that detects active caspase-3, but not the proenzyme, showing the presence of processed caspase-3 in nonactivated BMMCs, and depletion of cell-associated caspase-3 after mast cell degranulation using A23187. The specificity of the caspase-3 Ab was verified by inclusion of soluble peptide corresponding to the epitope used for immunization (peptide blocking; right panels). (C) After 4 h of incubation of control cells or cells treated with either calcium ionophore (A23187) or IgE receptor cross-linking (IgE-TNP/OVA), samples (50 μL) were collected from the medium and analyzed for caspase-3–like activity, in the absence or presence of either a caspase-3 inhibitor (Ac-DEVD-CHO) or a serine protease inhibitor (Pefabloc SC), as indicated. (D) Effect of mast cell activation by either IgE receptor cross-linking or calcium ionophore stimulation on expression of caspase-3 mRNA as measured by quantitative PCR analysis. (E) Control experiment showing robust β-hexosaminidase release in response to both A23187 and IgE receptor cross-linking. n = 3 independent experiments; duplicate measurements. (A, C) *p < 0.05, **p < 0.01, ***p < 0.001 compared with its noninhibited sample (i.e., A23187 + Ac-DEVD-CHO compared with A23187 alone); (B) *p < 0.05 compared with nonstimulated control samples. (E) *p < 0.05 compared with nonstimulated control cells.

Accumulation of active caspase-3 accompanies the differentiation of bone marrow precursor cells into mature mast cells
cells developed with either of the protocols used (Fig. 3C). Together, these data indicate that high caspase-3 mRNA expression and accumulation of high levels of active caspase-3 protein accompany mast cell maturation.

**Active caspase-3 is found within granule-like compartments in mast cells**

To address the subcellular localization of active caspase-3 in mast cells, we first analyzed for its presence in cytosolic extracts. A titration experiment showed that digitonin concentrations >2 μg/ml were sufficient to recover the cytosol marker LDH from the mast cells along with a general release of cellular protein (Fig. 4A). However, no detectable active caspase-3 was recovered at digitonin concentrations up to ≥20 μg/ml, thus arguing against its presence within the cytosolic compartment. In contrast, solubilization of active caspase-3 was seen when increasing the detergent concentrations ≥20 μg/ml (Fig. 4A). At these conditions, we also observed solubilization of CPA3, CPA3 being a mast cell–specific granule protease (19) (Fig. 4B), as well as solubilization of enzymatic activity toward Z-Phe-Arg-AMC, a substrate commonly used for detection of lysosomal cysteine cathepsin activity (Fig. 4A). These findings suggest that active caspase-3 is localized in mast cell compartments that have characteristics similar to those of the secretory granules/lysosomes.

To further analyze the subcellular localization of active caspase-3 in mast cells, we used a cell-permeable substrate (PhiPhiLux) to detect caspase-3 activity in live cells. As seen in Fig. 5A (upper panels showing a representative cell; additional stained cells are shown in Supplemental Fig. 1), staining of mast cells with PhiPhiLux revealed the presence of caspase-3–positive granule-like compartments, showing a predominant localization close to the cell membrane. Importantly, the active caspase-3–positive cells also stained positively for calcein-AM, the latter a marker for cellular viability (Fig. 5A). A Z stack showing the three-dimensional distribution of active caspase-3 in mast cells is displayed in Supplemental Fig. 2. The staining with PhiPhiLux was

**FIGURE 2.** Enzymatically active caspase-3 is found in PCMCs. PCMCs (4 × 10⁶ cells) were nontreated (control; Ct) or induced to degranulate using calcium ionophore (A23187) or IgE receptor cross-linking, as indicated. (A) Immunoblot analysis for active caspase-3 showing the presence of active caspase-3 in nonactivated PCMCs, and depletion of cell-associated active caspase-3 after mast cell degranulation using A23187, but not IgE receptor cross-linking. Quantification of the band intensities is shown in the right panel relative to control (set to 1). (B) Samples (50 μl) from the conditioned media were collected after 4 h of culture in the absence or presence of mast cell–activating stimulus, as indicated, and were assayed for caspase-3 activity. (C) Effect of IgE receptor cross-linking or calcium ionophore (A23187) stimulation on expression of caspase-3 mRNA in PCMCs, as measured by quantitative PCR analysis. (A) n = 3 independent experiments; (B) n = 4 independent experiments, duplicate measurements; (C) n = 3 independent experiments, duplicate measurements. *p < 0.05.

**FIGURE 3.** Expression of active caspase-3 protein and of caspase-3 mRNA is linked to mast cell maturation. Bone marrow cells were isolated and were differentiated into mast cell phenotype. (A) Caspase-3 mRNA levels in bone marrow cells at different stages of mast cell maturation. As indicated, mast cell maturation was induced using either conditioned medium from WEHI-3B cells (WEHI-CM; contains IL-3 and produces mast cells of connective tissue-like subtype), by a combination of IL-3 and SCF (alternative protocol resulting in mast cells of more mature connective tissue-like subtype), or by a combination of IL-3 + SCF + TGF-β + IL-9 (produces mast cells of mucosal-like subtype). (B) At the time points indicated, samples (corresponding to 0.3 × 10⁶ cells) were analyzed by immunoblot for procaspase-3 (upper panel) and for active caspase-3 (middle panel) protein. For comparison, cells were assessed for levels of mMCP-6, a mast cell–specific protease localized to secretory granules. Note that the appearance of active caspase-3 protein coincides with the appearance of mMCP-6, the latter a sign that the cells have acquired mature mast cell phenotype. (C) Immunoblot analysis showing the levels of active caspase-3 in mature (day 28) mast cells developed using different protocols, as indicated. The results are representative of two independent experiments.
completely abrogated after addition of a caspase-3 inhibitor (Fig. 5B, right panel). After activation of mast cells with calcium ionophore, the intracellular caspase-3–positive granule-like compartments disappeared (Fig. 5A, middle panels), along with the recovery of corresponding caspase-3–positive entities in the extracellular space (Fig. 5B, left panel). For a comparison, a cell with typical signs of apoptosis is depicted in Fig. 5A (lower panels), showing negativity for calcine-AM and intense caspase-3 activity throughout the entire cytoplasm. (B) The left panel shows a lower magnification of degranulated mast cells, where released caspase-3–positive compartments are seen in the extracellular space. The right panel depicts the abrogated PhiPhiLux staining after addition of caspase-3 inhibitor. Original magnification ×400.

To provide further insight into the subcellular location of active caspase-3 in mast cells, subcellular fractionation was performed using methodology previously established for purification of secretory granules from rat peritoneal mast cells (20). The granule-enriched fraction obtained contained undetectable levels of LDH activity, indicating that there was no significant contamination of cytosolic proteins in the granule fraction (Fig. 6A). Note that LDH activity results in NADH consumption and thus reduced absorbance from initial levels (indicated by a dashed line). The left bar (cells) represents LDH activity in sonicated cells, that is, the total activity of the cell preparation used for purification of granules. Moreover, there were negligible levels of histone deacetylase activity in the granule fraction, suggesting that the contamination by nuclear compounds was minimal (data not shown). In contrast, the granule fraction contained substantial levels of trypsin-like activity (Fig. 6B). As most of the trypsin-like activity in mast cells is due to granule-located trypase, this suggests that the purified...
fraction indeed is enriched with secretory granules. In further support for this, the granule fraction contained substantial levels of the granule-specific protein CPA3 as shown by immunoblot analysis (Fig. 6C). As seen in Fig. 6D, it was also evident that caspase-3 activity was detected in the granule-enriched fraction, and that caspase-3 protein was readily detected by immunoblot analysis (Fig. 6E). Hence, these data support that active caspase-3 is located in secretory granules of mast cells.

Secretory granule permeabilization causes release of active caspase-3 into the cytosol

The findings described above suggest that preformed active caspase-3 is present in granule-like compartments of mast cells. If so, disruption of the mast cell granule membrane would have the potential to result in release of active caspase-3 into the cytosol. To evaluate this possibility, we subjected mast cells to LLME, a lysosomotropic agent that has mast cell granule-permeabilizing properties (13, 21), and analyzed cytosolic extracts for the presence of active caspase-3. As shown in Fig. 7, LLME induced a robust and rapid release of active caspase-3–like activity (Fig. 7A, lower panel) and protein (Fig. 7B, upper panel) as well as release of Z-Phe-Arg-AMC–cleaving activity (Fig. 7A, upper panel) into the cytosol. Moreover, LLME induced a release of mast cell granule marker proteins (CPA3 and mMCP-6) into the cytosolic fraction (Fig. 7B, middle and lower panels, respectively), thus confirming its granule-permeabilizing properties. Together, these findings suggest that permeabilization of mast cell granules results in a rapid release of preformed active caspase-3 into the cytosolic compartment.

Discussion

The general notion is that activation of procaspase-3 to enzymatically active protease is an event mainly associated with cell death, and thus that caspase-3 is predominantly present as inactive proenzyme in viable cells. In this study, we present data challenging this notion by showing that enzymatically active caspase-3 is present in viable mast cells.

The presence of active caspase-3 in live mast cells was supported by the presence of caspase-3–like enzymatic activity and protein bands corresponding to processed caspase-3 in the absence of cell death–inducing agents. Importantly, active caspase-3 was found both in mast cells derived by in vitro differentiation of bone marrow precursors to mature mast cells (BMMCs) and in mast cells expanded from in vivo–differentiated mast cells (PCMCs), indicating that expression of active caspase-3 protein is a general property of mast cells. In strong support of the latter notion, we show that the appearance of active caspase-3 is strongly associated with maturation of bone marrow precursor cells into mature mast cells, whereas procaspase-3 was present in cells of all stages of differentiation. It was also intriguing to note that the mRNA levels for caspase-3 showed an increase as cells gained a mature mast cell phenotype, suggesting that high levels of caspase-3 mRNA expression are a phenotypic characteristic of mature mast cells.

An important issue to resolve was the intracellular location of active caspase-3. Normally, if procaspase-3 is activated within the cytosolic compartment, this will lead to apoptotic cell death. However, because our data suggested that active caspase-3 was in

![FIGURE 6.](http://www.jimmunol.org/) Active caspase-3 is recovered in granule-enriched fractions. Secretory granules were purified from BMMCs. (A) LDH activity (60 min) in sonicated cells used as starting material (cells) and in purified granules. Note that LDH activity results in decreased absorbance from initial level indicated by a dashed line, that is, LDH activity is undetectable in purified granules. (B) Trypsin-like activity (30 min) in starting material (Cells) and in purified granules. (C) Immunoblot analysis showing CPA3 levels in starting material (Cells) and in purified granules. The lower panel shows quantification of band intensities relative to control intensity (set to 1). (D) Casp3 Activity

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Caspase-3–like activity in starting material (Cells) and in purified granules (16 h). (E) Immunoblot analysis showing levels of active caspase-3 in starting material (Cells) and in purified granules. The lower panel shows quantification of band intensities. Experiments were performed in duplicates from two independent granule-enriched purifications.
granule permeabilization caused release of active caspase-3 into the cytosolic compartment. Indeed, secretory granules are released to the exterior of the cells. In several approaches, first, we subjected the mast cells to agents that cause mast cell degranulation, whereby the contents of their secretory vesicles distinct from those that are released by CD8+ T cells without signs of cell death (29). In another study, evidence was presented suggesting that caspase-3 released from mast cells may have a role in monocyte-to-macrophage differentiation (30).

Interestingly, whereas calcium ionophore stimulation resulted in robust release of caspase-3 accompanied by a reduction of intracellular caspase-3, IgE receptor cross-linking failed to induce significant caspase-3 release. Hence, caspase-3 appears to be present in secretory vesicles distinct from those that are released by IgE-mediated mechanisms. Clearly, this may be in line with recent studies that have identified granule subtypes of distinct contents in mast cells (31).

**Disclosures**
The authors have no financial conflicts of interest.
References

Supplementary Figure 1. Confocal microscopy analysis showing three representative caspase-3-positive mast cells (BMMCs). Caspase-3 activity was monitored by using a cell-permeable caspase-3 substrate (PhiPhiLux; red). Cell viability was monitored using Calcein-AM (green) and the nucleus is depicted by DAPI staining (blue).
Supplementary Figure 2. Confocal microscopy analysis showing a representative caspase-3-positive mast cell (BMMC). Caspase-3 activity was monitored by using a cell-permeable caspase-3 substrate (PhiPhiLux; red). Cell viability was monitored using Calcein-AM (green) and the nucleus is depicted by DAPI staining (blue). The figure depicts a Z stack of one single cell, with images collected with 2.3 µm intervals.

Suppl. Fig. 2