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The Cathelicidin LL-37 Activates Human Mast Cells and Is Degraded by Mast Cell Tryptase: Counter-Regulation by CXCL4

Florian Schiemann,* Ernst Brandt,* Roland Gross,* Buko Lindner,† Jessica Mittelstädt,* Christian P. Sommerhoff,‡ Jan Schulmistrat,* and Frank Petersen*

The cathelicidin LL-37 represents a potent antimicrobial and cell-stimulating agent, most abundantly expressed in peripheral organs such as lung and skin during inflammation. Because mast cells (MC) overtake prominent immunomodulatory roles in these organs, we wondered whether interactions exist between MC and LL-37. In this study, we show for the first time to our knowledge that physiological concentrations of LL-37 induce degranulation in purified human lung MC. Intriguingly, as a consequence LL-37 rapidly undergoes limited cleavage by a released protease. The enzyme was identified as β-tryptase by inhibitor studies and by comparison to the recombinant protease. Examining the resulting LL-37 fragments for their functional activity, we found that none of the typical capacities of intact LL-37, i.e., MC degranulation, bactericidal activity, and neutralization of LPS, were retained. Conversely, we found that another inflammatory protein, the platelet-derived chemokine CXCL4, protects LL-37 from cleavage by β-tryptase. Interestingly, CXCL4 did not act as a direct enzyme inhibitor, but destabilized active tetrameric β-tryptase by antagonizing the heparin component required for the integrity of the tetramer. Altogether our results suggest that interaction of LL-37 and MC initiates an effective feedback loop to limit cathelicidin activity during inflammation, whereas CXCL4 may represent a physiological counter-regulator of β-tryptase activity. The Journal of Immunology, 2009, 183: 2223–2231.

Mas cells (MC) are located in peripheral organs such as skin and lung, positioned in close vicinity to blood vessels and in contact with connective tissue or mucosal cells. Depending on the respective organ, MC show remarkable differences in responsibility to inflammatory mediators as well as in their content of releasable proteases (1). In general, MC are recognized as primary instigators of certain inflammatory responses, in particular immediate hypersensitivity reactions and anaphylaxis. The most relevant pathway to activate MC in allergy is the cross-linkage of FcεRI after allergen invasion. As a consequence, MC degranulate and release various preformed inflammatory mediators, such as certain cytokines, chemokines, lipid derivatives, and MC-specific proteases (1, 2). Among MC proteases, expression of the chymotryptic serine hydrolase chymase is predominantly limited to skin MC, whereas the tryptic enzyme β-tryptase is typical for MC from all tissues (1). However, as seen with MC from lung, there exist also chymase-positive cell populations in this organ, depending on their localization within the airways (3). Released as an active enzyme in a protease-proteoglycan complex, chymase has been discussed to exert mainly proinflammatory functions, as it was shown to proteolytically activate important mediators such as angiotensin, IL-18, or the neutrophil-attracting chemokine CXCL7 (4–7). An interesting example of how chymase proteolytic activity may be controlled is the processing of connective tissue-activating peptide CTAP-III, a platelet-derived precursor of CXCL7. Remarkably, generation of CXCL7 underlies the strict control of another platelet-derived chemokine CXCL4, which acts as a direct enzyme inhibitor of chymase (7). Whereas chymase is additionally susceptible to serine protease inhibitors from plasma and airway tissue to some extent, β-tryptase has been described to resist these macromolecular inhibitors (8). Interestingly, MC release β-tryptase as a highly active tetrameric molecule in complex with heparin, which renders the catalytic center of the enzyme inaccessible to the majority of the above-mentioned inhibitors (9, 10). Instead, other natural inhibitors such as lactoferrin have been identified that represent a quite different functional principle by acting as heparin antagonists, causing the decomplexation and subsequent disintegration of tetrameric β-tryptase into almost inactive monomers (11, 12). Similar to chymase, β-tryptase is being discussed to play a pivotal role in MC-dependent inflammatory responses by proteolytically modulating the activity of peptide mediators. Although several lines of evidence point out that β-tryptase is also involved in anti-inflammatory functions as exemplified by the decrease of lethality in an murine sepsis model as a consequence of proteolytic inactivation of IL-6 (13), most reports suggest a more proinflammatory role for this enzyme (14). The latter view is supported by in vivo findings that injection of β-tryptase in guinea pigs results in tissue invasion of granulocytes (15).

Lining the borders of different tissues, MC are exposed to potent inflammatory mediators and antimicrobial peptides that are secreted by invading leukocytes or by connective tissue and epithelial cells. Among antimicrobial peptides, cathelicidin constitutes a

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3 Abbreviations used in this paper: MC, mast cell; MS, mass spectrometry.

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prominent example of a molecule that is produced by many different cell types. In humans, cathelicidin is released as an inactive precursor (CAP-18) (16). This pro-peptide becomes immediately processed by neutrophils, the major producing cells, into a phylogenetically conserved N-terminal peptide termed cathelin and a species-specific 37 aa long C-terminal fragment LL-37 (17). Due to its strong cationic and amphipathic properties LL-37 displays a broad antimicrobial spectrum against Gram-positive and -negative bacteria (17). Apart from these properties, the immunoregulatory functions of LL-37 have become increasingly apparent during the last years, as exemplified by its ability to bind and neutralize LPS or to exhibit strong cell stimulatory activity on leukocytes, where it acts, for example, as a potent chemotaxin for T cells (18–20).

Even more interesting, LL-37 has been recently described to induce chemotaxis in rat MC and degranulation in the human MC line LAD-2 (21, 22). However, the activity of LL-37 on human primary MC has not been investigated so far.

As mentioned, MC may become exposed to high concentrations of cathelicidins during an inflammatory response. We therefore raised the question whether and how these two players might interact and regulate each other’s activity. In this study, we show for the first time to our knowledge that LL-37 induces degranulation in human primary MC. Conversely, MC rapidly degrade LL-37 into different fragments by their releasable protease β-tryptase. However, degradation products lack the microbicidal, LPS-neutralizing and cell-stimulatory capacity of intact LL-37. Looking for the regulator of cell action, degradation products were stored at −20°C. In some experiments anti-IgE-activated MC were incubated overnight with 1 µg/ml human monoclonal IgE derived from cell line U266 (Calbiochem). Biological responsibility of MC was routinely monitored by their capacity to degranulate in response to affinity-purified goat antiserum cross-linking human IgE (anti-IgE; BioSource International). Potential cytotoxic effects of stimuli were determined by using the UptiBlue reagent (Interchim) according to the recommendations of the manufacturer. Reduction of the oxidized indicator by mitochondrial enzymatic activity of the viable cells was analyzed colorimetrically for up to 5 h after stimulation (30).

**Materials and Methods**

**Reagents**

Peptide LL-37 was synthesized by solid-phase synthesis using an automatic peptide synthesizer (model 433A; Applied Biosystems) and purified by RP-HPLC with an C18 column using a linear gradient of 0–70% ace-tonitrile as described (23). Purity and integrity was confirmed by mass spectrometry (MS). The peptide was lyophilized and stored at −20°C until reconstitution in 0.1% acetic acid before use. Human natural CXCL4 was purified (>99%) in our laboratory from supernatants of thrombin-stimu-lated platelets as described (24). The inhibitors aprotinin, soybean trypsin inhibitor, chymostatin, leupeptin, and NFD49 were from Sigma-Aldrich, whereas APC 366 (HNAP, N-(1-hydroxy-2-naphthyl)-l-arginyl-l-prolin-amide) was obtained from Tocris. Recombinant human MC β-tryptase was expressed in *Pichia pastoris* and isolated by a modification of published methods (25, 26). The purity of the recombinant enzyme (>95%) was verified by SDS-PAGE and N-terminal amino acid sequencing (IVG GQEAPRS...). The specific activity, determined by both burst titration with 4-methylumbelliferyl p-guanadinobenzene and titration with synthetic inhibitors was 90% or more of the theoretical value (27, 28). Recombinant human β-tryptase was stabilized routinely with unfractionated heparin from porcine intestinal mucosa (Sigma-Aldrich) in a weight to weight ratio of 1:1. In some experiments, heparin of different molecular mass was used at the concentration indicated. Heparin with an average mass 15,000 (from bovine intestinal mucosa), 6000, and 3000 (both porcine intestinal mucosa) was obtained from Sigma-Aldrich.

**Preparation and purification of human lung MC**

For isolation of human lung MC, tumor-free lung tissue obtained from patients with bronchial carcinoma undergoing lobectomy was provided by the Section of Pathology (Research Center Borstel, Borstel, Germany). Approval for these studies was obtained from the institutional review board at the University of Lübeck (Lübeck, Germany) and informed consent was provided according to the declaration of Helsinki.

Lung specimens were chopped into pieces and placed overnight in ice-cold MC buffer (7). To disperse cells enzymatically, lung pieces were incubated in MC buffer containing 0.1% gelatin, 1.5 mg/ml dispase II, 0.375 mg/ml chymopapain (both from Sigma-Aldrich), 0.75 mg/ml collagenase type I, 1.79 mg/ml elastase (both Worthington Biochemical) under agitation for 4 h at 37°C. Purification of lung MC was performed as described elsewhere in detail for skin MC (7). Briefly, after enrichment of MC by Percoll gradient centrifugation, further purification was achieved by immunofinity magnetic enrichment, using anti-PE-conjugated microbeads (Miltenyi Biotec) in combination with PE-conjugated mAb 97A6 (IOTest) specific for CD203c, an Ag exclusively expressed on MC and basophils (29). MC purity in these preparations ranged from 78 to 90% as assessed by toluidine blue staining. Viability was always >85% as assessed by trypan blue exclusion.

**Cell culture**

Purified MC (5 × 10⁶/ml) were cultured in Stempro medium (Life Technologies) containing 2 mM l-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin (Biochrom) and 100 ng/ml human recombinant stem cell factor (PeproTech). Medium was changed every week. The purity of MC used for experiments ranged from 88 to 100% as assessed by toluidine blue staining. Viability was always >85% as assessed by trypan blue exclusion. To facilitate equal loading with IgE before biological assays, MC were incubated overnight with 1 µg/ml human monoclonal IgE derived from cell line U266 (Calbiochem). Biological responsibility of MC was routinely monitored by their capacity to degranulate in response to affinity-purified goat antiserum cross-linking human IgE (anti-IgE; BioSource International). Potential cytotoxic effects of stimuli were determined by using the UptiBlue reagent (Interchim) according to the recommendations of the manufacturer. Reduction of the oxidized indicator by mitochondrial enzymatic activity of the viable cells was analyzed colorimetrically for up to 5 h after stimulation (30).

**Processing of LL-37 by MC and β-tryptase**

For LL-37 processing by MC, the indicated number of cells was either preincubated (30 min for 37°C) with processing buffer alone (PBS-Dulbecco0.1% BSA) or buffer containing 3 µg/ml anti-IgE. Thereafter, 100 µl of cell suspension or cell-free supernatant were mixed with 100 µl of PBS-Dulbecco0.1% BSA and incubated for 30 min at 37°C before the addition of LL-37 to characterize the processing enzyme involved. For processing assays, recombinant human β-tryptase was incubated at the concentrations indicated for different time periods at 37°C with 3 µM LL-37.

**MC degranulation assay**

To quantify the MC degranulating activity of LL-37 and LL-37 fragments, the supernatant of activated MC was tested for released β-hexosaminidase activity as described (7, 31). Briefly, prewarmed MC (2.5 × 10⁶/ml) were activated with LL-37, LL-37 fragments, or anti-IgE for 30 min at 37°C in PBS-Dulbecco0.1% BSA. Thereafter, 50 µl of serially diluted cell-free supernatants were incubated with 50 µl of β-hexosaminidase substrate 4-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (4 mM in citrate buffer (pH 4.5)) for 18 h at 37°C. The reaction was terminated by addition of 50 µl of glycine buffer (pH 10.3) and liberated p-nitrophenol measured at A 405 nm. Degranulation is given as proportion of released to total β-hexosaminidase activity as calculated from cell lysates run in parallel.

**Electrophoresis and Western blot analysis of cathelicidin**

SDS-PAGE and immunoblotting was performed as described elsewhere (32, 33). Blinding of blotting membranes and incubation steps with Abs were performed using Roti-Immunoblock buffer (Carl Roth). Blots were stained using rabbit polyclonal antiserum rabbit anti-LL-37 (Innovagen) and IRDye800-conjugated goat anti-rabbit antiserum (1/5000; Rockland). Bands were visualized and quantified by an Odyssey infrared imaging system (LI-COR Biosciences).

**Analysis of LL-37 cleavage products by HPLC and MS**

For analysis of LL-37 fragmentation, samples were separated by RP-HPLC using a C2/C18 column (µRPC PC3.25/0) on a SMART chromatography unit (Amersham Pharmacia). The column was developed with a linear ace-tonitrile gradient (0–50%), and eluting peaks were detected at 214 nm wavelength. Eluting proteins were collected, and their mass determined by MS using MALDI TOF-MS (ReflexIII; Bruker-Daltonics) as described elsewhere (7, 34).
**Microbicidal assay**

Microbicidal activity of untreated LL-37 or LL-37-treated with active or inactive β-tryptase was quantified by liquid microbicidal assay with slight modifications (35). Tryptase was inactivated by adjusting TFA to a concentration of 0.1% to the sample. The final concentration of TFA in biological assays did not exceed 0.02% and did not affect LL-37-mediated biological functions. Suspensions of Escherichia coli 2131 or Staphylococcus aureus 42D provided by T. K. Lindhorst (University of Kiel, Kiel, Germany) and S. A. Zaat (University of Amsterdam, Amsterdam, the Netherlands), respectively, were cultured to early log-phase in trypticase soy broth (Difco). Bacteria were inoculated at 1 × 10⁶ CFU/ml in 10 mM sodium phosphate buffer (pH 7.2) containing 0.06% trypticase soy broth. LL-37 samples serially diluted in 0.01% acetic acid (5 μL) were mixed with bacterial suspensions (45 μL) under agitation for 2 h at 37°C. Thereafter, suspensions were quantitatively plated in triplicates onto trypticase soy broth agar. Microbicidal activity was assessed the next day after counting CFUs.

**Dendritic cell culture**

Monocytes were isolated by counterflow elutriation of mononuclear cells prepared by density centrifugation of citrate-buffered peripheral blood of healthy donors (36). These cells were differentiatated to dendritic cells in the presence of GM-CSF and IL-4 (each 500 U/ml) in RPMI 1640 containing 10% FCS. Half of the medium was exchanged every 2–3 days. Cells were harvested after 5–7 days and seeded at a density of 1 × 10⁶ cells/ml. Thereafter, cells were stimulated with or without LPS (10 ng/ml) in the presence or absence of 3 μM LL-37 (either pretreated with active or inactivate β-tryptase or left untreated) for 18 h. TNF and IL-12p70 concentrations in cell-free supernatants were determined by quantitative ELISA (Natu Tec and R&D Systems).

**Determination of β-tryptase activity**

Tryptase activity was determined by conversion of the chromogenic substrate Bzl-Phe-Val-Arg-p-nitroanilide as described (37). A total of 50 μL of β-tryptase (0.5 μg/ml) in complex with heparin were incubated with 50 μL of 10 mM Bzl-Phe-Val-Arg-p-nitroanilide in 0.1 M Tris-HCl (pH 8.3) at room temperature. Liberated nitroanilide was measured spectrophotometrically at 405 nm every 2 min. Results are the ΔOD per minute as calculated from the first 6 min of the reaction (linear increase in OD). Alternatively, β-tryptase activity was determined spectrofluorometrically, using tos-Gly-Pro-Arg-AMC as described (28).

**Statistical analysis**

Data are presented as mean ± SD for the number of experiments indicated. Statistically significant (p < 0.05) differences among the treatment groups were calculated using Student’s paired t test.

**Results**

**LL-37 induces MC activation**

The activation of cells of the human MC line LAD-2 by the cathelicidin LL-37 having been described before (22), we wondered whether LL-37 would also induce degranulation in human primary MC. Therefore, isolated lung MC were incubated with increasing dosages of synthetic LL-37 and cell degranulation was quantified by determining β-hexosaminidase activity released into the supernatant. In these experiments LL-37 dose-dependently induced significant MC degranulation at concentrations from 3 μM and higher, increasing to ~60% of total cell content in the presence of 10 μM (Fig. 1A). MC release was not caused by a cytotoxic effect of LL-37. This finding became evident from a cytotoxicity assay in which cells treated with LL-37 up to 10 μM showed no difference in their metabolic activity as compared with untreated cells (data not shown). As higher concentrations of LL-37 (from 30 μM on) affected viability of the cells, a dosage of 3 μM was used in additional experiments. Having seen that LL-37 is able to degranulate lung MC, we next examined whether the cells conversely would act on LL-37 in terms of modifying the peptide. In a first approach, we incubated 3 μM LL-37 alone or with 1000 or 10,000 MC/ml for 30 min and subjected the cell-free supernatant to separation by RP-HPLC to assess changes in LL-37 interactions with MC.
quantity. In samples in which LL-37 was incubated in the absence of cells, the peptide eluted as a single major peak at a retention time of 31 min (Fig. 1B). However, LL-37 was undetectable after incubation with either 1000 or 10,000 cells/ml. Suspecting that this result was due to proteolytic degradation by released MC proteases, we examined cell-free supernatants of anti-IgE-stimulated MC for their impact on LL-37. As it turned out, LL-37 exposed to supernatants equivalent to 1000 cells/ml appeared slightly reduced and completely disappeared at an MC concentration of 10,000 cells/ml (Fig. 1C). This suggested that activated MC indeed secretes LL-37 modifying mediators and raised the question whether LL-37 as an activator of MC degranulation would perhaps induce its own degradation by released proteases. To clarify this question, we incubated lung MC with LL-37 in the presence or absence of an inhibitor of Gs proteins, NF449, which was identified as an effective inhibitor of LL-37-induced MC degranulation in a separate study (F. Schiemann, unpublished observations). For analysis samples were subjected to SDS-PAGE, Western blotting, and staining with LL-37-specific rabbit serum. As seen in Fig. 1D, LL-37 alone migrated as one major band, whereas upon exposure to MC, this band became significantly reduced by more than 65% (as determined by scanning analysis). Several immunoreactive bands corresponding to lower molecular size became visible. Most interestingly, in the presence of NF449, the effect of MC was drastically reversed and nearly 70% of the LL-37 remained intact, whereas 0.1% DMSO (solvent control) had no effect. These results suggest that cell activation and most likely degranulation were necessary for the modification of LL-37. The appearance of immunoreactive molecules smaller than LL-37 following MC exposure strongly suggested the formation of LL-37 degradation products, supporting our view that LL-37 might cause its degradation by stimulating MC release.

Proteolytic cleavage of LL-37 by MC is mediated by β-tryptase

Further support for the ability of MC to proteolytically degrade LL-37 was provided by the emergence of several smaller peaks that eluted during RP-HPLC separation of cell-free supernatants at shorter retention times (Fig. 2A). To identify the protease responsible for degradation of LL-37, fragments obtained after complete degradation of the intact peptide with MC for 30 min were isolated by RP-HPLC (Fig. 2A, elution profile, upper and middle) and their respective mass (Da) was determined. The majority of masses found by MS could be clearly assigned to distinct sequences within the primary structure of the full-size peptide (Fig. 2B). Most interestingly, LL-37 became cleaved at several positions C terminally to lysine and arginine residues, revealing a cleavage pattern characteristic of trypsin digestion. As MC tryptase represents the most prominent protease exhibiting cleavage specificity C-terminal to positively charged amino acids, we hypothesized that this enzyme should be predominantly responsible for processing of LL-37. To test this theory, LL-37 was incubated with recombinant β-tryptase under conditions identical with those applied in the corresponding experiments with MC. Similarly, the resulting elution profile of these samples revealed complete degradation of full-size LL-37 and a comparable pattern of peaks, indicating the formation of fragments corresponding to those obtained by digestion with MC (Fig. 2A, elution profile, bottom). In fact, MS analysis of the purified peptides revealed their identity to those obtained by MC degradation of LL-37 (Fig. 2B). In control experiments using β-tryptase inactivated before the assay by acidification with 0.1% TFA, the peak for full-size LL-37 remained unchanged and no cleavage products were observed (data not shown). These data as well as our observation that several other MC proteases (chymase and cathepsin G, data not shown) were unable to process LL-37 strongly support our hypothesis of β-tryptase representing the LL-37 cleaving protease in MC. To further substantiate these data, we performed experiments using a panel of MC enzyme inhibitors, in which β-tryptase and MC were incubated with inhibitors before the addition of LL-37. Cell-free supernatants were then analyzed for the presence of LL-37 by Western blotting using LL-37-specific Ab. Tryptase-mediated processing of LL-37 (Fig. 2C, top) became expectedly inhibited by leupeptin, HNAP, and protamine, whereas cathepsin G and chymase inhibitors aprotinin, soybean trypsin inhibitor, and chymostatin were ineffective in this respect. In corresponding experiments with MC (Fig. 2C, bottom), we observed an identical inhibition pattern, i.e., effective blockade of
processing by leupeptin, HNAP and protamine, whereas the other inhibitors remained ineffective. Altogether these results clearly show that β-tryptase is the major LL-37 processing enzyme in MC.

Processed LL-37 lacks typical cathelicidin activities
After having identified the enzyme responsible for LL-37 degradation as well as the resulting cleavage products, we next investigated the consequences of processing for typical cathelicidin functions. First, we assessed the capacity of processsed LL-37 to activate MC degranulation. Therefore, LL-37 was incubated with buffer alone or with recombinant β-tryptase for 30 min. Complete degradation of LL-37 in the latter sample was routinely verified by analysis of products by RP-HPLC (data not shown). To exclude potential side effects by heparin or residual impurities contained in the protease preparation, the activity of LL-37 incubated with acidified (i.e., inactivated) β-tryptase was tested in parallel. Active β-tryptase alone (10 μg/ml) had no effect on MC degranulation (data not shown). Expectedly, untreated LL-37 dose-dependently induced degranulation from 0.3 μM to higher doses (Fig. 3), whereas in sharp contrast, no degranulation was observed with processed LL-37 even at concentrations equivalent to 3 μM. Controls with samples receiving inactive β-tryptase were unaffected. Taken together, these data show that fragments resulting from β-tryptase-mediated processing of LL-37 do not retain the capacity to induce MC degranulation.

Apart from an inducer of cellular effects, LL-37 has been mainly recognized as an efficient antimicrobial agent (17). Thus, the same samples were tested for MC degranulating activity were examined in a liquid microbicidal assay using Gram-negative (E. coli) as well as Gram-positive (S. aureus) bacteria. Intact LL-37 impaired growth of E. coli as well as S. aureus within a concentration range from 0.1 to 3 μM of the peptide (Fig. 4). However, with both germs tested, LL-37 digested with β-tryptase completely failed to inhibit bacterial growth, whereas the corresponding control with inactivated protease was fully active. Thus, β-tryptase-mediated processing of LL-37 drastically impairs its microbicidal activity (by at least 30-fold), if not abolishes it completely.

CXCL4 regulates β-tryptase activity
As described, degradation of LL-37 by MC is a rapid process. This process may require effective regulatory mechanisms to avoid inappropriate clearance of cathelicidin at the site of inflammation. Because we found previously that the platelet-derived chemokine...
CXCL4 represents a strong inhibitor of MC chymase (7), we wondered whether CXCL4 would also counter-regulate β-tryptase proteolytic activity. Consequently, we analyzed processing of LL-37 by MC in the absence and presence of increasing concentrations of CXCL4. Western blot analysis of the resulting products revealed that in the absence of CXCL4, LL-37 became expectedly degraded by MC (Fig. 6A). However, the presence of CXCL4 effectively protected LL-37 from degradation in a dose-dependent manner resulting in inhibition of processing by 70% at 12 μM and by 58% at 4 μM of the chemokine, as determined by scanning analysis of the blot. To distinguish whether the effect of CXCL4 is due to interaction with the MC or with released enzyme, we performed a corresponding assay using recombinant β-tryptase (Fig. 6B). Comparable to the effect observed with intact MC, degradation of LL-37 was dose-dependently reduced by increasing concentrations of CXCL4 with a maximal inhibition of 84% at 12 μM and 79% inhibition observed at 4 μM. These results clearly show that protection of LL-37 by CXCL4 is based on the direct interaction of the chemokine with the catalyzing enzyme and is not mediated through MC. To further substantiate this result, we tested whether CXCL4 would also inhibit the cleavage of a small chromogenic peptide substrate by β-tryptase. As shown in Fig. 6C, the chemokine dose-dependently reduced the cleavage of the peptide substrate, effecting half-maximal inhibition at 0.4 μM CXCL4 and reaching a plateau of 10% enzyme activity at chemokine concentrations from 1.2 μM to higher doses. These data show that CXCL4 acts as a direct and potent inhibitor of β-tryptase.

Next we focused our interests on the mechanism underlying the inhibitory function of CXCL4. Because several known naturally occurring inhibitors of β-tryptase act by destabilizing its active tetrameric conformation, we hypothesized that CXCL4 might operate in a similar way. As CXCL4 is capable of binding glycosaminoglycans with high affinity, we assumed that the chemokine
imal inhibition was already reached at 15 nM CXCL4, a 10-fold increase over the control. The mechanism behind this phenomenon is discussed later.

Interestingly, theCXCL4 concentration required for half-maximal inhibition was seen at 0.1 μg/ml heparin of m.w. 3000, whereas at 0.05 μg/ml heparin the IC50 value increased to 1.2 μM. These results indicate that CXCL4 indeed inhibits β-tryptase by acting as a heparin antagonist. Interestingly, a 4-fold molar excess of CXCL4 over high-molecular-weight heparin (m.w. 15,000, as used in this experiment) was required for evolving inhibitory effects. Repeat- ing these experiments using heparin with average mass of 3000 and 6000, respectively, revealed that the molar ratio between CXCL4 and heparin strongly depends on the size of the glycosaminoglycan. In the presence of 0.1 μg/ml heparin of m.w. 6000 an IC50 of 14 nM was observed, whereas in the presence of 15 μg/ml heparin of m.w. 3000, half-maximal inhibition was seen at 310 nM CXCL4 (Fig. 7B). As derived from these data, the CXCL4 to heparin molar ratio required for inhibition drastically decreases to 0.9 and 0.06 with heparin of m.w. 6000 and 3000, respectively. The mechanism behind this phenomenon is discussed later.

**Discussion**

Although cross-linking of cell surface high affinity FcεRI is the prime mechanism for activation of MC in allergy, MC may also become activated by a number of intrinsic mediators derived from various cell types. Challenged by reports by others describing that a human MC line underwent activation in response to the cathelicidin LL-37, we examined whether this response would also occur for human primary MC (22). Most intriguingly, we did not only find that LL-37 in a range of concentrations between 1 and 10 μM induces degranulation in human lung MC, but also that LL-37 thereby initiates its own degradation by a MC-released protease. These concentrations of LL-37 appear to be physiologically relevant because lavage fluids, which represent highly diluted material, constitutively contain cathelicidins between 0.1 and 0.2 μg/ml. Under inflammatory conditions, like chronic obstructive pulmonary disease or cystic fibrosis, peptide concentrations up to 2 μg/ml can be observed (41, 42). Proteolytic cleavage of the cathelicidin was extremely efficient and rapid, as exemplified by the total cleavage of substrate (3 μM LL-37) within 30 min by 1000–10,000 MC/ml. Although there exist many examples that MC convert inactive precursor peptides to potent inflammatory mediators, like CTAP-III into CXCL7, C3 into C3a, and prochymosin into chemerin, only few authors reported of MC stimulating their own inactivation by proteolytic processing, such as C3a, substance P, and vasoactive intestinal peptide (7, 43–47). Regarding our findings that LL-37 similarly induces its own degradation by initiating MC degranulation, evidence is growing that MC-mediated cleavage may play a dual role during the course of inflammation, i.e., as an instigating as well as a limiting process. According to a very recent publication by Di Nardo et al. (48), murine MC produce and proteolytically process the precursor CAP-18. However, the major CAP-18-derived cleavage product IGE24, generated upon activation with IL-4, retained microbicidal activity in contrast to the peptides generated by human MC from LL-37. This result could be due either to differences in human and murine MC proteases or to differences in the accessibility of substrates CAP-18 and LL-37 to these proteases. If the latter were true, this difference would support a dual role for MC, mediating activation and inactivation of certain substrates in sequence, as previously reported for C3.

In this study, we show for the first time to our knowledge that human MC proteolytically cleave exogenous LL-37 into various fragments of different size, encompassing at least six peptides of 18 to 80 aa in lengths. These conditions are different from those found in neutrophils that were demonstrated to release the precursor CAP-18, which then becomes cleaved into LL-37 by extracellular neutrophil proteinase 3 (17). Because LL-37 undergoes no further degradation by neutrophils, it may serve as a substrate for processing by other cell populations besides exhibiting its direct cell stimulatory and antimicrobial activities. Evidence comes from studies by others reporting the presence of cathelicidin fragments in skin of two C-terminal peptides (IK14, NL8) that we also found upon MC-mediated cleavage of LL-37, whereas still other fragments have been found in human sweat (49, 50). Intriguingly, all cleavage products we could identify in MC supernatants exhibited various m.w. we also found upon MC-mediated cleavage of LL-37, whereas still other fragments have been found in human sweat (49, 50). Intriguingly, all cleavage products we could identify in MC supernatants exhibited enzyme activity in contrast to the peptides generated by human MC from LL-37. This result could be due either to differences in human and murine MC proteases or to differences in the accessibility of substrates CAP-18 and LL-37 to these proteases. If the latter were true, this difference would support a dual role for MC, mediating activation and inactivation of certain substrates in sequence, as previously reported for C3.
5 and other trypsin proteases were identified to be involved in LL-37 processing. Regarding this result, one may speculate that processing by trypsin enzymes could represent a general principle in the regulation of LL-37 availability. However, there apparently exist dramatic differences concerning the activity of the individual cleavage products. We found that LL-37 fragments generated by MC lost microbicidal activity against E. coli and S. aureus as well as their MC-stimulating capacity. By contrast, LL-37 peptides resulting from processing by either human skin proteases or human sweat are reported to possess significantly increased microbicidal activity against E. coli and S. aureus, although in the latter study the cell-stimulatory capacity was found to be slightly impaired. These striking differences in functional activities may relate to differences in molecular size existing between the cleavage products from MC and sweat or skin. Whereas long LL-37 fragments like KR20, KS30, and RK31 or KR20, LL23, KS27, and KS30 have been found in human sweat or human skin, respectively, the fragments generated by human lung MC from proteolysis of exogenous LL-37 were significantly smaller (maximum 18 aa, Fig. 2B). In line with our findings, the authors reported that such small cleavage products lack biological activity. Thus, although trypsin enzymes in general may represent key regulators of LL-37 availability, the individual enzymes as well as specific conditions in tissues may differentially modulate the activity of LL-37.

At first view it may appear incomprehensible why activated MC should degrade an antimicrobial peptide in an allergic or inflammatory situation. However, LL-37 does not only play a role in the direct combat of bacterial invaders, but also regulates activity of inflammatory situation. However, LL-37 does not only play a role in the general impact of the chemokine on MC protease activity, studies demonstrating that MC proteases are capable of activating as well as deactivating inflammatory stimuli (as seen, for example, with CXCL7 and LL-37). However, the intriguing two-step process initiates that the chemokine also acted as an inhibitor for β-trypetase (34). As a consequence, it appears that CXCL4 could regulate the overall protease activity of MC. Because in allergen-challenged mice MC activation coincides with platelet aggregation in the surrounding vasculature, it is tempting to speculate that this chemokine has its role in dampening and locally limiting MC enzyme activity during inflammatory situations (57). Moreover as demonstrated by our studies, the inhibitory capacity of CXCL4 is not limited to certain substrates, but provides extended protection for other substrates as documented by its inhibitory capacity for the cleavage of small synthetic peptide substrates (7). Regarding this general impact of the chemokine on protease activity, studies addressing the role of CXCL4 during MC-driven inflammatory responses in vivo are mandatory.

Irrespective of the regulatory role of CXCL4, our findings demonstrate that MC proteases are capable of activating as well as deactivating inflammatory stimuli (as seen, for example, with CXCL7 and LL-37). However, the intriguing two-step process initiated by LL-37, consisting in MC activation followed by rapid MC-mediated degradation of the stimulus in return, can be assigned a dead-end route in which the activating agent is finally removed and no longer available for autocrine and paracrine activation of cells.

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References


