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Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37

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Summary

Effectors of the innate immune system, the antibacterial peptides, have pivotal roles in preventing infection at epithelial surfaces. Here we show that proteinases of the significant human pathogens *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis* and *Streptococcus pyogenes,* degrade the antibacterial peptide LL-37. Analysis by mass spectrometry of fragments generated by *P. aeruginosa* elastase *in vitro* revealed that the initial cleavages occurred at Asn-Leu and Asp-Phe, followed by two breaks at Arg-Ile, thus inactivating the peptide. Proteinases of the other pathogens also degraded LL-37 as determined by SDS-PAGE. *Ex vivo*, *P. aeruginosa* elastase induced LL-37 degradation in human wound fluid, leading to enhanced bacterial survival. The degradation was blocked by the metalloproteinase inhibitors GM6001 and 1, 10-phenantroline (both of which inhibited *P. aeruginosa* elastase, *P. mirabilis* proteinase, and *E. faecalis* gelatinase), or the inhibitor E64 (which inhibited *S. pyogenes* cysteine proteinase). Additional experiments demonstrated that dermatan sulphate and disaccharides of the structure [ΔUA(2S)-GalNAc(4,6S)], or sucroseoctasulphate, inhibited the degradation of LL-37. The results indicate that proteolytic degradation of LL-37 is a common virulence mechanism and that molecules which block this degradation could have therapeutic potential.

Introduction

Epithelial surfaces of various organisms are continuously exposed to bacteria. During recent years the innate immune system, based on antibacterial peptides originally described in silk worms (Steiner *et al*., 1981), has been attributed important roles in the initial clearance of bacteria at biological boundaries susceptible to infection (for references see reviews by Selsted and Ouellette, 1995; Lehrer and Ganz, 1999; Schröder and Harder, 1999; Boman, 2000). Cathelicidins, consisting of a conserved N-terminal domain and a C-terminal antibacterial region, constitute one family of such peptides. In humans, the cathelicidin hCAP-18 is processed by proteinase 3 (a serine proteinase) to generate the active peptide LL-37, which exerts antibacterial activity against both Gram-negative and Gram-positive bacteria (Sörensen *et al*., 2001). LL-37 has been isolated from neutrophils (Zanetti *et al*., 1995) and subpopulations of lymphocytes and monocytes (Agerberth *et al*., 2000). This peptide is also found in seminal plasma (Malm *et al*., 2000), in the lung (Bals *et al*., 1998; Agerberth *et al*., 1999), and in keratinocytes during inflammation (Frohm *et al*., 1997). Furthermore, it has been reported that LL-37 has synergistic effects with α-defensin, another major antibacterial peptide found in the azurophilic granules of neutrophils (Nagaoka *et al*., 2000). The significance of cathelicidins for bacterial clearance is exemplified by recent findings indicating that the mouse antibacterial peptide CRAMP protects the skin from invasive bacterial infection (Nizet *et al*., 2001).

Pathogens, however, appear to overcome the innate immune defence systems giving rise to various infections. In some conditions, such as chronic ulcers of the skin, bacteria such as *P. aeruginosa*, *E. faecalis,* and *P. mirabilis,* manage to infect and persist for long periods of time. During infection, these pathogens, which are frequently found in chronic ulcers (*P. aeruginosa*; 20–30%, *E. faecalis* 80%, *P. mirabilis* 15%) (Hansson *et al*., 1995), use a combination of virulence strategies. For instance, release of various proteinases, such as elastase and alkaline proteinase of *P. aeruginosa* (Morihara *et al*., 1965; Fukushima *et al*., 1989; Okuda *et al*., 1990), may modulate host responses involving kallikreins, coagulation factors, complement, cytokines, and antiproteinases (for a review, see Travis *et al*., 1995). Interestingly, degradation of complement, antiproteinases, and matrix components *in vitro* by the proteinases, closely resembles the

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degradation pattern of these molecules seen in wound fluid *in vivo* (Schmidtchen, 2000, and unpublished data).

The well known bacterial persistence in chronic skin ulcers and the fact that antiproteinase deprivation and uncontrolled proteolysis characterize these ulcers raise the possibility that bacterial proteinases may affect antibacterial peptides. In this study, data are presented showing that bacterial proteinases, such *as P. aeruginosa* elastase, rapidly degrade and inactivate LL-37, leading to enhanced bacterial survival. Furthermore, we show that this proteolytic degradation may be modulated by proteinase inhibitors and by sulphated poly- and di-saccharides.

Results

Bacterial proteinases degrade and inactivate LL-37

To examine the bactericidal effects of intact LL-37, the activity of the peptide against *S. pyogenes*, *E. faecalis, P. mirabilis* and *P. aeruginosa* was determined. The bacterial strains were separately incubated with 0–20 μg ml^{-1} of LL-37. *Pseudomonas aeruginosa*, *E. faecalis, P. mirabilis,* and *S. pyogenes* were effectively killed by the peptide at or above concentrations of 2–10 μg ml^{-1} (Fig. 1). We then investigated the effects of *P. aeruginosa* elastase on LL-37. Initial experiments indicated that the peptide was rapidly degraded, with no detectable intermediate peptides on SDS-PAGE (not shown). Thus, a titration was performed and proteinase amounts yielding detectable amounts of LL-37 degradation products were used in further experiments. The addition of 30 mU of proteinase (~25 ng protein) to 10 μg peptide (molar ratio enzyme to peptide ~1:2500) yielded intermediate fragments of approximately 3–4 kDa after an incubation period of 1–30 min (Fig. 2A, upper panel). Longer incubations yielded no visible peptides in the SDS-PAGE system used (16.5%). The proteinase amounts used correspond to enzyme levels found in ~5–10 μl of conditioned media from a *P. aeruginosa* culture grown overnight in TH-medium (as determined by an azocasein assay). Next, the effect of these LL-37 intermediates was investigated. In bactericidal assays, *E. faecalis* bacteria were incubated with the peptide fractions (corresponding to 10 μg ml^{-1}), and those containing the 3–4 kDa forms (Fig. 2A, 5–30 min incubations) exerted residual bactericidal activity (40–60% bacteria killed), whereas subsequent incubations (1–20 h) contained no such activity (not shown). The finding that the 30 min incubation, which was devoid of intact peptide, exerted residual activity (~40% of control), suggests that the larger ~4 kDa degradation product is bactericidal. Finally, structural analysis of LL-37 fragments generated by *P. aeruginosa* elastase was performed. Liquid chromatography–mass spectrometry time of flight (LC-MS TOF) and MS-MS TOF analysis of the material corresponding to the 1 min to 1 h degradations (Fig. 2A) yielded major peptides of masses 3708.859, 3178.637, 2822.450, 2326.169 and 1401.748 Da. A correspondence between the peptide intensities of the major 3–4 kDa bands on electrophoresis (Fig. 2A) and the peak heights on mass spectrometry was noted (not shown). The *P. aeruginosa* cleavage points were determined and are indicated (Fig. 2B). Interestingly, the proteinase acted on a region of LL-37 that has been attributed with antibacterial activity (Oren *et al.*, 1999).

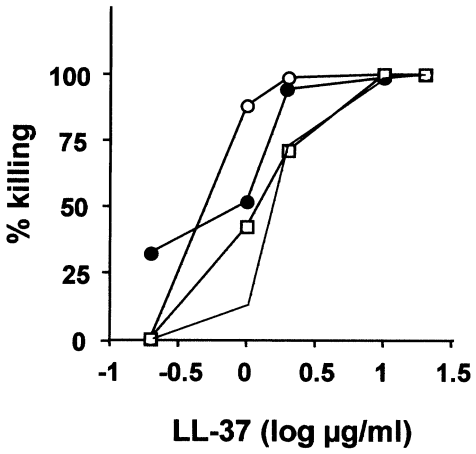

Fig. 1. Bactericidal effect of LL-37. 2×10^6 colony-forming units (CFU) ml^{-1} of *E. faecalis* (□), *S. pyogenes* (○), *P. mirabilis* (–), or *P. aeruginosa* (●) were incubated with LL-37 at indicated peptide concentrations for 2 h (*S. pyogenes, P. mirabilis* and *E. faecalis*) or 4 h (*P. aeruginosa*). CFUs were determined, and representative experiments are shown.

Having proven that fragmentation of LL-37 leads to loss of antibacterial activity (using *E. faecalis* as the test organism), we examined whether proteinase production was protective for *P. aeruginosa*. In this context, various experimental approaches were tested. Analyses of *P. aeruginosa* growth in TH or LB medium before and after addition of proteinase inhibitors (GM6001 or 1, 10-phenantroline), showed that these, at levels necessary for inhibition of elastase (50–100 μM), inhibited bacterial growth, which prevented us from performing MIC analyses under conditions necessary for *P. aeruginosa* growth. The effect of supplementing *P. aeruginosa* with proteinase was therefore investigated. Addition of elastase, corresponding to the concentrations found in overnight cultures of *P. aeruginosa*, resulted in significant reduction of LL-37-mediated bacterial killing (Fig. 2C and D). Next, to show that degradation abolished bacterial binding, the uptake of fluorescent labelled LL-37 by bacteria was studied using fluorescence microscopy and flow cytometry. As shown in Fig. 3B, Texas red-conjugated LL-37 rapidly bound to and stained *P. aeruginosa* bacteria. After an incubation of the labelled LL-37 with *P. aeruginosa* elastase, no binding of LL-37 to the bacteria could be detected (Fig. 3D). Both

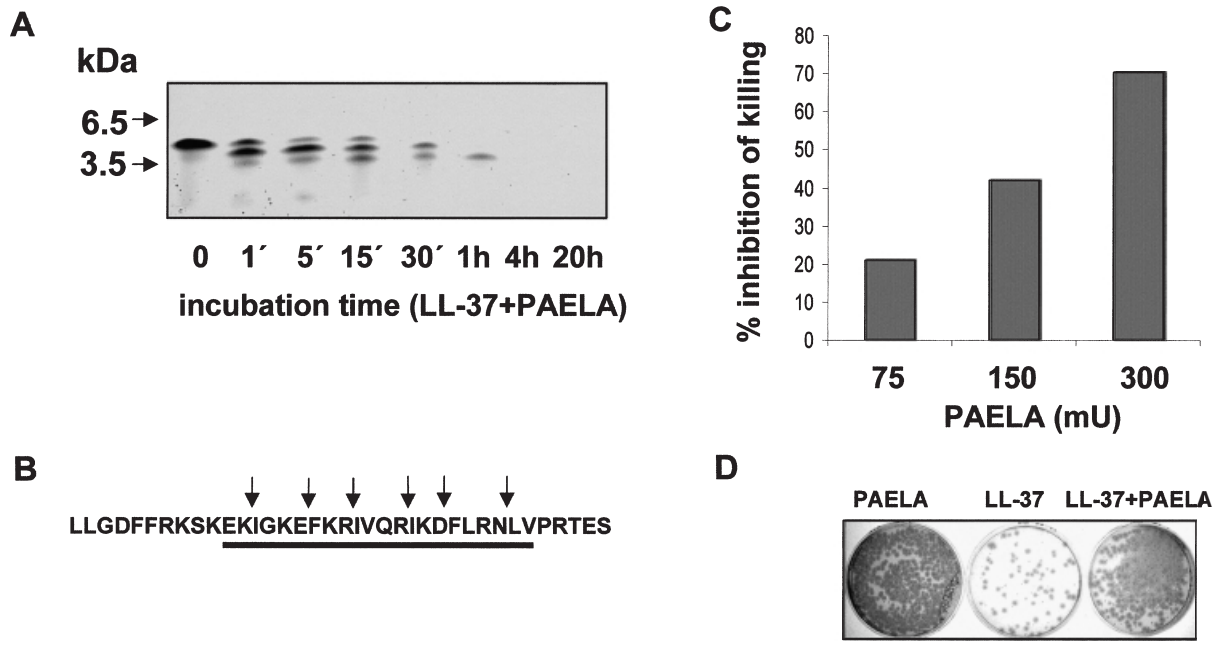

Fig. 2. *Pseudomonas aeruginosa* elastase degrades and inactivates LL-37.
A. LL-37 (10 μg) was incubated with 30 mU of *P. aeruginosa* elastase (PAELA) for various periods of time (shown on the *x*-axis). Equal aliquots of the incubations were then analysed by SDS-PAGE (16.5% Tris-tricine gel). A volume corresponding to 2 μg of LL-37 was loaded on the gels. Molecular mass markers are indicated to the left.
B. The LL-37 degradation products were analysed by mass spectrometry and the cleavage points are indicated. The proposed antibacterial region of LL-37 is underlined below.
In C and D, the bactericidal effect of LL-37 in the absence or presence of PAELA was determined.
C. 2×10^6 colony-forming units (CFU) ml^{-1} of *P. aeruginosa* were incubated with PAELA only (75, 150 or 300 mU), with LL-37 (0.2 μg) only, or with PAELA (70, 150 or 300 mU) and LL-37 (0.2 μg) at 37°C for 4 h (50 μl reactions). Plates were streaked and CFUs determined, and the inhibition of LL-37 mediated bacterial killing by added PAELA is indicated. A representative experiment (of three) is shown.
D. The inset illustrates the experiment in C using 150 mU of PAELA, 0.2 μg LL-37, or 150 mU PAELA and 0.2 μg LL-37 (PAELA + LL-37). 150 mU amounts to the PAELA secreted in 50 μl by overnight *P. aeruginosa* cultures.

incubations were analysed directly and, hence, identical amounts of Texas red label were added in both cases to the bacteria (this was easily verified by UV-illumination). The images in Fig. 3B and D were recorded using identical settings for the microscope and CCD camera software. To quantify the fluorescence more stringently, we analysed the fluorescence distribution of bacteria incubated with intact labelled LL-37 (Fig. 3E, rightmost histogram) and bacteria incubated with LL-37 that had been subjected to prior treatment with *P. aeruginosa* elastase (Fig. 3E, leftmost histogram). Non-degraded LL-37 gave rise to a distinct fluorescence, whereas the fluorescence histogram of bacteria incubated with degraded LL-37 did not differ from that of unlabelled bacteria (not shown).

Finally, the effects of other bacterial proteinases on LL-37 were investigated. Incubation of LL-37 with *E. faecalis* growth medium (containing gelatinase), as well as purified *S. pyogenes* cysteine proteinase yielded similar fragments as those generated by *P. aeruginosa* elastase, as determined by SDS-PAGE. *Proteus mirabilis* proteinase did not generate these intermediates. However, for all bacteria, longer incubation periods yielded almost complete digestion of LL-37 (Fig. 4A). Incubation of LL-37 with wound fluid from a patient with a *P. aeruginosa* infected chronic ulcer, also yielded almost complete degradation. Fluid from a surgical (sterile) wound did not exhibit this effect (Fig. 4B). Analysis of additional acute (three samples) and chronic, infected, wound fluids (two samples) from other patients yielded similar results (not shown).

Ex vivo *experiments using human wound fluid and neutrophil extracts*

The finding that wound fluid from infected ulcers degrades LL-37, prompted us to further investigate LL-37 degradation in wound fluids, and the effects on bacterial survival of *P. aeruginosa* proteinase production in these wound fluids. Various ulcer-derived *P. aeruginosa,* producing either undetectable levels of elastase or expressing high levels of elastase (three isolates each, of which one from each group is presented in Fig. 5, see also Schmidtchen *et al.*, 2001a) were grown in TH medium or in wound fluid

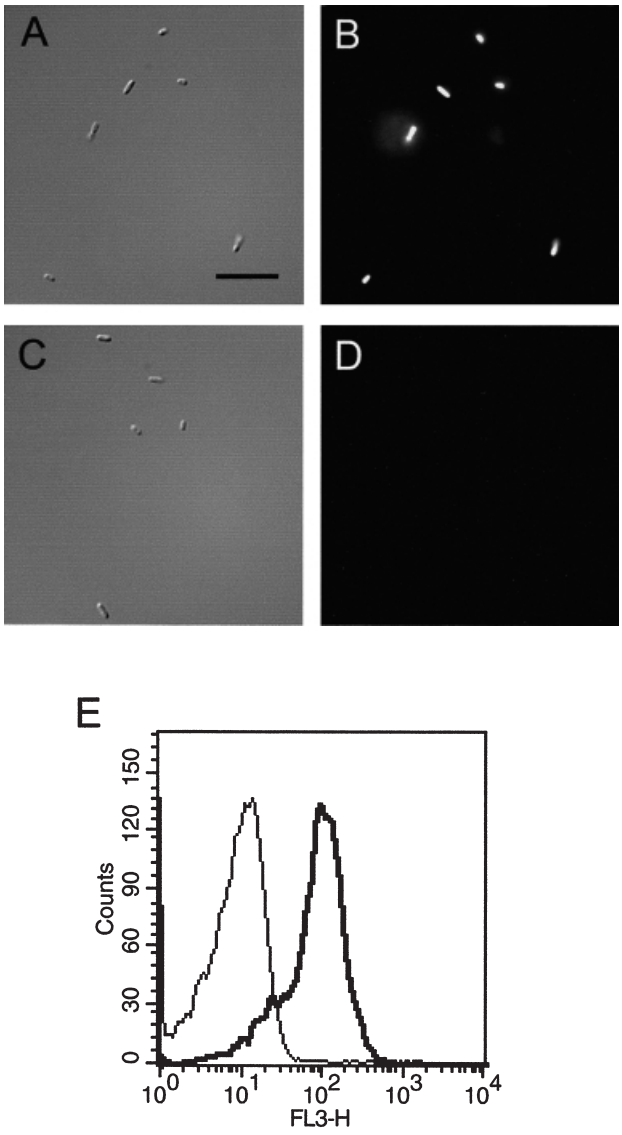

Fig. 3. Binding of Texas red-labelled LL-37 to bacteria is abolished by *Pseudomonas aeruginosa* elastase. B shows the red fluorescence of bacteria (5×10^6 ml^{-1}) stained with intact Texas red-LL-37 (10 μg ml^{-1}) and D shows bacteria stained with Texas red-LL-37 subjected to prior treatment with *P. aeruginosa* elastase. Images in B and D were recorded using identical instrument settings. The corresponding Nomarski images are shown in A and C. E shows the staining of bacteria quantified by flow cytometry. The rightmost histogram shows bacteria labelled with intact Texas red-LL-37, whereas the leftmost histogram shows bacteria labelled with the degraded peptide. Results shown are representative of three separate experiments.

obtained from sterile surgical wounds. *Pseudomonas aeruginosa* elastase was only detected (by zymographic analysis and immunoblotting) in supernatants from cultures of previously characterized elastase-expressing bacteria, and the levels of elastase were similar in TH and in wound fluid (Fig. 5A). The minor 50 kDa enzyme is alkaline proteinase (Schmidtchen *et al.*, 2001a). Sterile wound fluid alone contained the human 92 and 72 kDa gelatinases (MMP-9 and MMP–2 respectively) (Fig. 5A). Having confirmed elastase production, the various wound fluids were then incubated with Texas red-labelled LL-37. As shown in Fig. 5B, significant degradation of LL-37 was only noted in the wound fluid containing elastase-producing *P. aeruginosa* (ELA +). Thus, the data show that wound fluid infected *ex vivo* with elastase-producing *P. aeruginosa*, has the capacity to rapidly degrade LL-37. On the basis of these observations, we examined whether the different *P. aeruginosa* isolates, when grown in wound fluid, exhibited variable sensitivity to exogenously added LL-37. In growth assays, overnight cultures of *P. aeruginosa* were inoculated (5%) in 200 μl of 10% human wound fluid (in phosphate-buffered saline). After an incubation period of 5 h, 0–30 μg of LL-37 was added and bacterial growth assessed (by measuring absorbance at 490 nm). Five and 10 μg LL-37 had no effect relative the controls (not shown). Whereas the elastase-producing *P. aeruginosa* was unaffected by LL-37 at all concentrations (in contrast to the results with the same isolate in the bactericidal assays shown in Fig. 1), the non-producing isolate was inhibited by LL-37 doses at or above 20 μg (100 μg ml^{-1}) (Fig. 5C). This concentration of LL-37 exceeded the bactericidal concentration of the peptide (obtained in 10 mM Tris-buffer; 10–20 μg ml^{-1}, see Fig. 1), indicating the presence of inhibitory substances in wound fluid, as previously described for plasma (Wang *et al.*, 1998). These results were similar for all other isolates studied, showing that soluble elastase promotes bacterial survival in wound fluid *ex vivo*. The fact that neutrophils secrete hCAP18 which is processed into active LL-37, raised the question whether the intact proprotein is affected by *P. aeruginosa* proteinase. To address this, human neutrophil extracts were treated with conditioned medium from elastase-producing or elastase-deficient *P. aeruginosa* (Fig. 6). As shown by immunoblotting using polyclonal antibodies against LL-37, degradation of hCAP18 was only noted after treatment with elastase-producing *P. aeruginosa*. Likewise, addition of purified *P. aeruginosa* elastase to neutrophil extracts resulted in a complete degradation of hCAP18 and LL-37 (not shown). As assessed by zymography, the neutrophil extracts contained several gelatinases, similar to those detected in acute wound fluid, corresponding to neutrophil-derived MMP-2 and MMP-9 (not shown). Analogously to the results with LL-37 in wound fluid alone (Fig. 5A), very little degradation of hCAP18 was seen in the control incubations.

Identification of substances that inhibit the degradation of LL-37

To find out whether the degradation of LL-37 could be

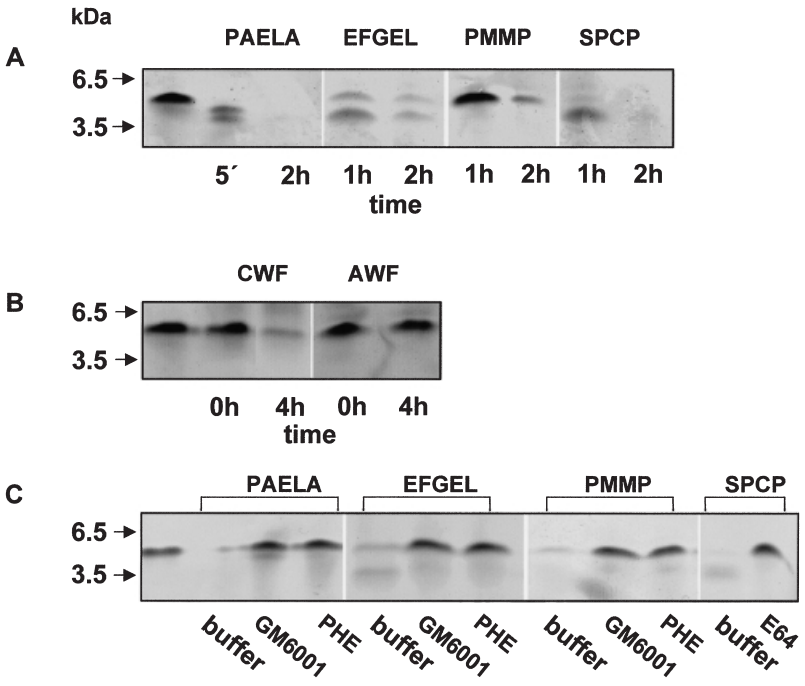

Fig. 4. Bacterial proteinases and chronic wound fluid degrade LL-37. LL-37 alone is run in the far left lanes of panels A–C.
A. LL-37 (1 μg) was incubated with *P. aeruginosa* elastase (PAELA), *E. faecalis* growth medium containing gelatinase (EFGEL), *P. mirabilis* growth medium containing a 50 kDa metalloproteinase (PMMP), or *S. pyogenes* cysteine proteinase (SPCP) at the indicated periods of time. The proteinase activity added (as determined by an azocasein assay) corresponded to 6 mU (PAELA) and 3 mU (for the other proteinases) per 10 μl reaction.
B. LL-37 (1 μg) was incubated for 4 h at 37°C with chronic (CWF) or acute (AWF) wound fluid (7% in phosphate-buffered saline in a total volume of 15 μl) respectively.
C. LL-37 (1 μg) was incubated with PAELA (12 mU), or with 6 mU each of EFGEL, PMMP, SPCP for 1 h in the absence or presence of inhibitors. GM6001; 100 μM GM6001, PHE; 2 mM 1, 10-phenantroline, E64; 10 μM E64. The material was analysed by SDS-PAGE on 16.5% Tris-tricine gels. Molecular mass markers are indicated to the left.

inhibited, the effects of proteinase inhibitors were studied. Results showed that degradation was abolished by the metalloproteinase inhibitors GM6001 and 1, 10-phenantroline (which both inhibited *P. aeruginosa* elastase, *E. faecalis* gelatinase and *P. mirabilis* proteinase) (Fig. 4C, PAELA, EFGEL and PMMP), or by the inhibitor E64 which inhibits the *S. pyogenes* cysteine proteinase (Björck *et al*., 1989) (Fig. 4C, SPCP). Previous studies showed that the antibacterial peptide α-defensin specifically bound to glycosaminoglycans, preferably chondroitin sulphate B (CS-B), and that this binding destroyed the antibacterial effect of the peptide (Schmidtchen *et al*., 2001). Therefore, we investigated whether LL-37 could bind to various glycosaminoglycans. As assessed by slot-binding experiments, LL-37 preferably bound to CS-B and CS-E (Fig. 7A), and subsequently, in a functional assay, we found that the various glycosaminoglycans blocked the bactericidal effects of the peptide (Fig. 7B). An octasulphated disaccharide, sucrosoctasulphate (SOS), also blocked the bactericidal activity of LL-37. Having shown that LL-37 binds to sulphated polysaccharides, we wanted to investigate whether these polysaccharides, their constituting disaccharides or related carbohydrates, exerted protective effects on LL-37 *vis-à-vis* added bacterial proteinase. Indeed, intact polysaccharides, preferably CS-B and CS-E, were able to protect LL-37 from degradation by *P. aeruginosa* elastase (Fig. 7C). Interestingly, a protective effect was noted after addition of trisulphated CS-disaccharides of the structure ΔUA(2S)-GalNAc(4,6S) (Fig. 7D), which are found (as co-polymers) in the CS-B fraction used here (Rodén *et al*., 1973). Notably, a related disaccharide (ΔUA(3S)-GalNAc(4,6S)) was recently identified in CS-E (Kinoshita *et al*., 2001). Sucroseoctasulphate also exhibited similar protective effects at almost equimolar concentrations of disaccharide relative the peptide (Fig. 7E). Sucrose did not exhibit these effects (not shown). These results show that anionic molecules of various sulphation patterns inhibit the degradation of LL-37. No effect on elastase activity (as determined by an azocasein assay) by either the CS variants or sucroseoctasulphate was detected (not shown).

Discussion

Bacterial evasion of peptide-based innate defences may prove to be important for the initial colonization of epithelial surfaces. Recently, pathways aimed at inactivating the innate immune system have been uncovered. For example, proteinases of common pathogenic bacteria, such as *P. aeruginosa*, *E. faecalis* and *S. pyogenes* were shown to release dermatan sulphate chains, leading to inactivation of the antibacterial peptide α-defensin (Schmidtchen *et al*., 2001b). Independent findings showed that the metalloproteinase lasA from *P. aeruginosa* induced release of another sulphated polysaccharide, heparan sulphate, leading to inactivation of innate defence and increased mortality during *P. aeruginosa* infection (Park *et al*., 2001). Staphylococcal resistance to defensins has recently been related to modification of bacterial membrane lipids with

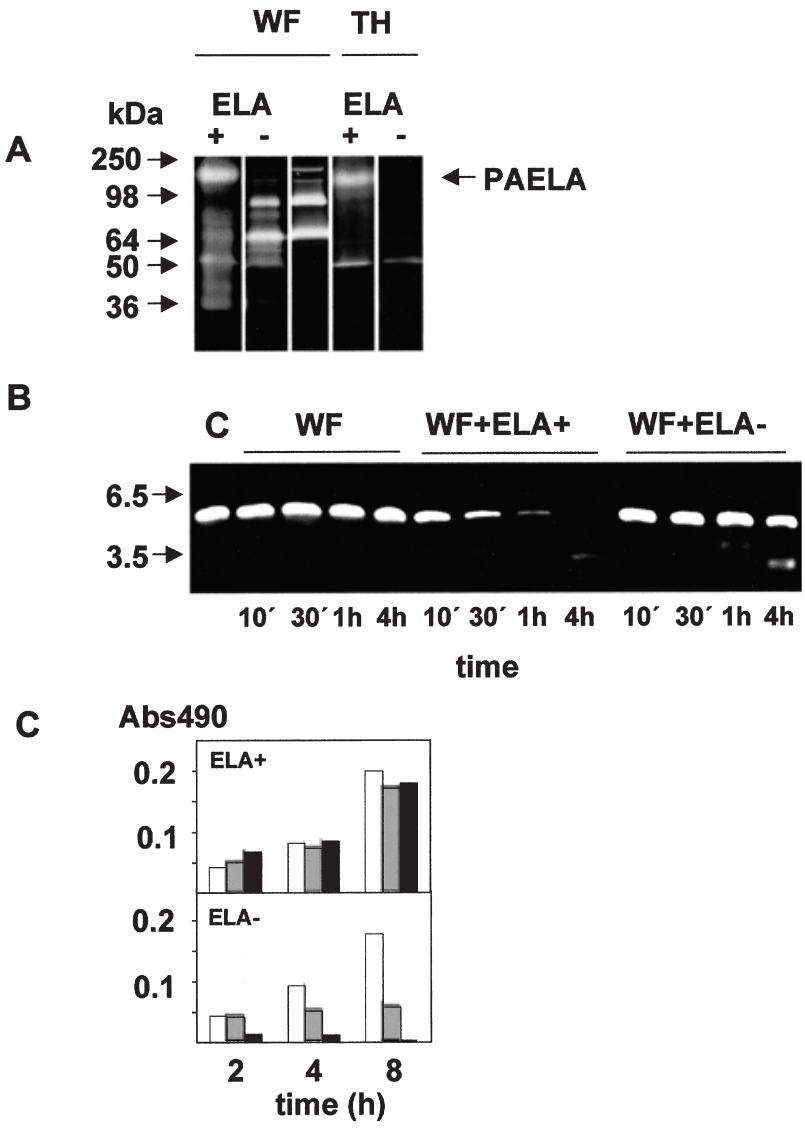

Fig. 5. *P. aeruginosa* elastase is secreted in human wound fluid, leading to degradation of LL-37 and enhanced bacterial survival.
A. Elastase-producing *P. aeruginosa* (ELA+) and an isolate devoid of elastase expression (ELA–) were grown to stationary phase in TH, or in wound fluid (WF) from surgical wounds. Then 0.5 μl of the material was analysed by zymography (10% polyacrylamide gels with 1 mg ml^{-1} gelatin). *P. aeruginosa* elastase (PAELA), which migrates as a ~150 kDa multimer on zymograms (Schmidtchen *et al.*, 2001a), is indicated to the right.
B. Texas red-labelled LL-37 (6 μg) was incubated with 5 μl of 50% sterile or infected wound fluids for the indicated periods of time. The material was analysed by SDS-PAGE on 16.5% Tris-tricine gels. WF, sterile wound fluid; WF + ELA+, wound fluid infected with elastase producing *P. aeruginosa*; WF + ELA–, wound fluid infected with *P. aeruginosa* with no detectable elastase expression. In A and B, molecular mass markers are indicated to the left.
C. Elastase-producing and elastase-deficient *P. aeruginosa* isolates (ELA+ and ELA–, respectively) were inoculated in 200 μl of 10% wound fluid, 0, 5, 10, 20 or 30 μg of LL-37 was added after an incubation period of 5 h, and bacterial growth was measured (as absorbance at 490 nm) from this time point. The data represent the mean of double samples, and a representative experiment (of three) is shown. No difference was noted between 0, 5 and 10 μg of LL-37 and the results using 10 (empty bars), 20 (dashed bars), or 30 μg LL-37 (black bars) are shown.

L-lysine (Peschel *et al.*, 2001). During *Shigella* infections, it was shown that this pathogen downregulates the expression of LL-37 and human β-defensins, with bacterial DNA as a potential regulator (Islam *et al.*, 2001). Additional acterial defence mechanisms directed against antibacterial peptides may involve modifications of outer cell wall components, such as lipopolysaccharide (Ernst *et al.*, 1999) or phosphorylcholine (Lysenko *et al.*, 2000) and the modulation of efflux pumps (Shafer *et al.*, 1998).

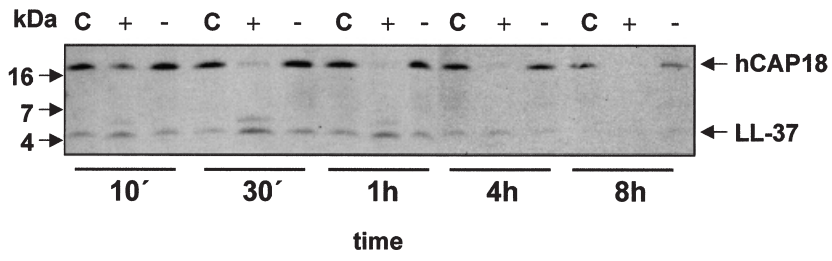

Fig. 6. hCAP18/LL-37 from neutrophil extracts is degraded by elastase-producing *P. aeruginosa*. Neutrophil extracts (5 μl corresponding to ~5×10^4 cells) were incubated with conditioned TH medium from elastase-producing or elastase-deficient *P. aeruginosa* isolates (+ and –, respectively, on top of panel). TH (C) was used as control. The material was run on 16.5% Tris-tricine gels after transfer of proteins to PVDF, immunoblotting was performed using polyclonal antibodies against LL-37. The positions of hCAP18 and LL-37 are indicated to the right and molecular mass markers (kDa) are indicated to the left.

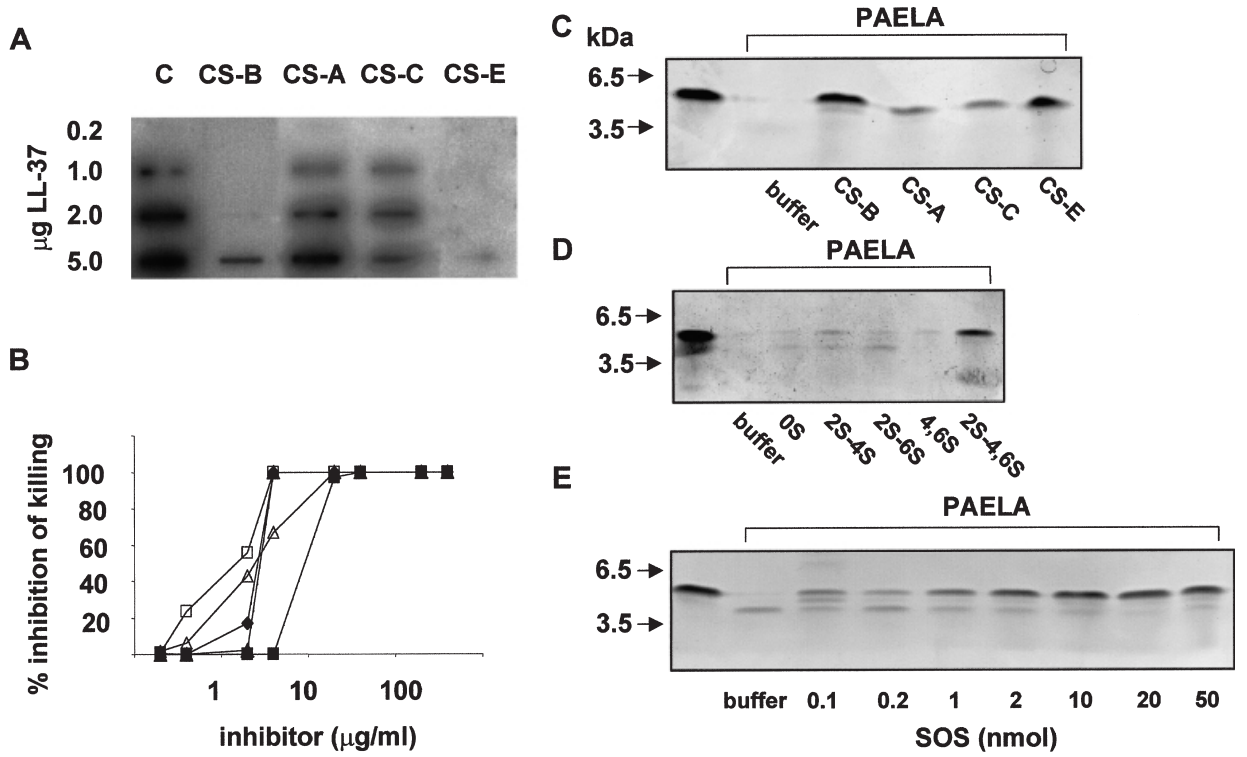

Fig. 7. Sulphated carbohydrates bind to and inhibit LL-37 and protect the peptide from *P. aeruginosa* elastase-mediated degradation.
A. LL-37 (0.2–5 mg) was applied onto nitrocellulose filters which were incubated with iodinated CS-B (~10 mg ml^{-1}). A buffer control (C) and different polysaccharides (2 mg ml^{-1}) were added for competition of binding; CS-B, A, C, E (chondroitin sulphate B, A, C, and E) followed by autoradiography.
B. For analysis of possible inhibition of LL-37 activity, 2×10^6 CFU ml^{-1} *E. faecalis* were incubated with LL-37 (10 μg ml^{-1}) for 2 h in the presence of the glycosaminoglycans CS-B (◆), CS-A (■), CS-C (●), CS-E (■), or the disaccharide sucroseoctasulphate (△) at the indicated concentrations. Colony-forming units were determined and representative experiments (of three) are shown.
C–E. LL-37 (1 μg) was incubated with *P. aeruginosa* elastase (12 mU) in a total of volume of 15 μl for 1 h without or together with inhibitor. In C, the inhibitors were: buffer; the glycosaminoglycans CS-B (chondroitin sulphate B); CS-A (chondroitin sulphate A); CS-C (chondroitin sulphate C); and CS-E (chondroitin sulphate E). In D, the inhibitors were: buffer; the disaccharides 0S; ΔUA-GalNAc; 2S-4S [ΔUA(2S)-GalNAc(4S)]; 2S-6S [ΔUA(2S)-GalNAc(6S)]; 4,6S [ΔUA-GalNAc(4,6S)]; and 2S-4,6S [ΔUA(2S)-GalNAc(4,6S)] (50 nmoles of disaccharide per reaction). In E, the inhibitors were buffer and sucroseoctasulphate (SOS) of the indicated amounts. The material was analysed by SDS-PAGE (16.5% Tris-tricine gels). Molecular mass markers (kDa) are indicated to the left, and LL-37 alone is run in the far left lanes.

Here, we show for the first time that proteinases of the clinically significant bacterial species *P. aeruginosa, E. faecalis, P. mirabilis* and *S. pyogenes* degrade the major human antibacterial peptide LL-37 and that this degradation leads to loss of LL-37 binding to bacteria and, ultimately, abolished bacterial killing. *Pseudomonas aeruginosa* elastase was shown to specifically hydrolyse LL-37. The P1′ position was preferentially occupied by hydrophobic amino acids (L, I, F), whereas the P1 position was less specific (N, D, R, E, K). The P1′ specificities corresponded well with those reported for the M4 peptidase family (thermolysin family) to which *P. aeruginosa* elastase belongs. *Enterococcus faecalis* gelatinase, as well as the 50 kDa metalloproteinase of *P. mirabilis* are also members of this group, having similar specificities (Rozalski *et al*., 1997; de Kreij *et al*., 2000). In this context, it should be noted that, in addition to cationic amino acids, most antibacterial peptides contain a significant (~50%) portion of hydrophobic amino acids (Hancock and Scott, 2000). Therefore, considering the data discussed above, and taking the specificities of the M4 peptidase family into account, it is conceivable that bacterial proteinases may have profound effects on the actions of antibacterial peptides. This notion is further supported by the fact that human α-defensin was also degraded by *P. aeruginosa* elastase (unpublished results).

From the perspective of chronic ulcers and considering recent observations identifying defensins in wound fluids (Frohm *et al*., 1996), as well as findings showing that cutaneous injury induces the release of LL-37 in response to infection (Dorschner *et al*., 2001), it is still an enigma how certain pathogens, such as *P. aeruginosa, E. faecalis* or *P. mirabilis* may persist and colonize for long periods of time in these ulcers. In this study, we present several lines

of evidence indicating a pivotal role for *P. aeruginosa* elastase in the evasion of LL-37 mediated bacterial killing. First, the finding that elastase production made *P. aeruginosa* completely resistant to the action of LL-37 during growth in human wound fluid *ex vivo* suggests that during *P. aeruginosa* infection of human wounds, release of elastase inactivates LL-37 and protects bacteria from the action of the antibacterial peptide. Second, *P. aeruginosa* elastase also induced degradation of the proprotein hCAP18 in neutrophil extracts. Third, we found that chronic wound fluid collected from *P. aeruginosa* infected leg ulcers, but not acute sterile wound fluid, had the capacity to degrade LL-37. Taken together, these findings correspond well with recent data showing that chronic venous ulcers are devoid of LL-37 and contain low levels of hCAP18, in contrast to normally healing wounds (Frohm-Nilsson, 2001). Because all chronic ulcers contain bacteria able to secrete proteinases, it is plausible that the observed lack of LL-37/hCAP18 is due to degradation by bacterial proteinases.

From a therapeutic point of view, inhibition of bacterially induced proteolysis could lead to increased levels of LL-37 *in vivo*. As hCAP-18 is processed to LL-37 by extracellular cleavage with proteinase 3, a serine proteinase (Sörensen *et al*., 2001), the use of specific metalloproteinase inhibitors, such as GM6001, could represent a possible treatment option in future *in vivo* studies. Considering the action of glycosaminoglycans and sulphated disaccharides, their effects also merits some consideration. The fact that these molecules block the bactericidal effects of LL-37, as well as protect the peptide from proteolysis, highlights the complexity and sometimes dual roles of bacterial virulence mechanisms. For example, it has been shown that bacterial proteinases are able to release CS-B, which may both inhibit (Schmidtchen *et al*., 2001b) and, as shown here, block antibacterial peptide degradation induced by the same proteinases. Other peptides, such as the antibacterial and highly cationic peptide PR-39, induce synthesis of the proteoglycan syndecan (Gallo *et al*., 1994), which, in turn, has the capacity to inactivate the peptide (Park *et al*., 2001). It is also worth considering that the functions of CS-B and the other glycosaminoglycans may go beyond mere inhibition of antibacterial activity. Recent data show that components of innate immunity, such as α–defensin, PR-39 and LL-37, act as potent chemoattractants (Huang *et al*., 1997; Yang *et al*., 2001). Hence, glycosaminoglycan-induced stabilization of these cationic peptides could be of biological significance in a highly proteolytic environment, perhaps underlying the herein noted remarkable stability of LL-37 in non-infected human wound fluids (which are rich in neutrophil elastase and various MMPs). In this context, it can be noted that growth factors, such as basic fibroblast growth factor, are bound to and protected from proteolysis by sulphated carbohydrates, including glycosaminoglycans and sucroseoctasulphate (Folkman *et al*., 1991; Klagsbrun, 1992), and that non-infected human wound fluid contains significant levels of CS-B (Penc *et al*., 1998).

In summary, we have presented data showing that the major human antibacterial peptide LL-37 is degraded and inactivated by proteinases of several significant human pathogens. This observation and the finding that proteinase inhibitors, glycosaminoglycans and sulphated disaccharides protect LL-37 from proteolytic degradation could be utilized in the search for novel antibacterial strategies.

Experimental procedures

Materials

LL-37, LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES (MW 4492.08) and Texas-red labelled LL-37 were synthesized by Innovagen AB, Lund. The Texas-red label including an additional cysteine was coupled to the aminoterminus of LL-37. The purity and molecular weight of these peptides was confirmed by mass spectral analysis (MALDI.TOF Voyager). Both peptides exhibited identical bactericidal activities using *E. faecalis* as the test bacterium. 1, 10-Phenantroline, GM6001, E64, the chondroitin sulphate (CS) preparations CS-A, -C, and -E, and the CS and dermatan sulphate (DS) disaccharides ΔUA-GalNAc, ΔUA(2S)-GalNAc(4S), ΔUA(2S)-GalNAc(6S), ΔUA-GalNAc(4,6S), ΔUA(2S)-GalNAc(4,6S) were purchased from Sigma. CS-B (DS36) was obtained from Dr L.-Å. Fransson. The preparation and characteristics of this fraction have been described previously (Rodén *et al*., 1973; Fransson *et al*., 1979). Sucroseoctasulphate (potassium salt, MW 1287.55) was purchased from Toronto Research Chemicals. *Pseudomonas aeruginosa* elastase was purchased from Calbiochem. In some experiments, we used an equivalent elastase enzyme, generously supplied by Dr H. Maeda, Kumamoto University, Japan.

Bacterial culture and proteinases

Pseudomonas aeruginosa, *E. faecalis,* and *P. mirabilis* isolates were initially obtained from chronic venous ulcers and were grown for 18 h at 37°C (stationary phase) in Todd–Hewitt (TH) medium. The *S. pyogenes* strain AP1 (40/58) was from the World Health Organization Collaborating Centre for References and Research on Streptococci (Prague, Czech Republic), and was grown to stationary phase in C-medium (Gerlach *et al*., 1983). Bacteria were pelleted by centrifugation and supernatants sterile-filtered (0.3 μm) and stored at –20°C. The cysteine proteinase of *S. pyogenes* was purified according to previous protocols (Herwald *et al*., 1996). Growth culture supernatants from the *E. faecalis* (expressing gelatinase) and *P. mirabilis* strains (expressing a 50 kDa metalloproteinase) (not shown) were sterile-filtered and used directly. The activity of the proteinase preparations was determined using an azocasein assay (Okamoto *et al*., 1997). For preparation of infected wound fluids, wound fluid from surgical wounds (1 ml diluted 1:1 with TH, denoted 50% WF in the text) was inoculated with 20 μl of overnight cultures of

P. aeruginosa. Bacteria were pelleted by centrifugation and supernatants were stored at −20°C.

Wound fluids

Wound fluids (100–600 μl) from patients with chronic, infected, venous ulcers were collected under a Tegaderm dressing for 2 h as previously described (Schmidtchen, 2000). Sterile wound fluid was obtained from surgical drainages after mastectomy. Collection was for 24 h after operation. Wound fluids were centrifuged, aliquoted and stored at −20°C. The use of this material was approved by the Ethics Committee at Lund University (LU 509–01, LU 708–01). Informed consent was obtained from the patients.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed using the Ready Gel system (Bio-Rad) on 16.5% precast polyacrylamide gels with a 4% stacking gel (Tris-tricine Ready Gel, Bio-Rad). For analysis of LL-37 degradation, peptides solubilized in 10 mM Tris, pH 7.4, were incubated with sterile or infected wound fluids, purified proteinases and, when indicated, additional substances were added (see figure legends for details). The incubations were stopped by boiling (5 min) and samples were supplemented with Tricine sample buffer (Bio-Rad). After fixing, the gels were stained with 0.25% Coomassie brilliant blue, destained and dried between two cellophane sheets in a Gel air dryer (Bio-Rad), and scanned (Duoscan T1222; Agfa). Texas red-labelled LL-37 was visualized directly after electrophoresis by the use of UV-illumination and digital photography. Pictures were processed using the Adobe photoshop software (Adobe).

Zymography

Wound fluids and bacterial supernatants were mixed with sample buffer (0.4 M Tris HCl, 20% glycerol, 5% sodium dodecyl sulphate (SDS), 0.03% bromophenol blue, pH 6.8) and electrophoresed on 10% polyacrylamide gels (1 mg of bovine gelatine per ml of gel). To remove SDS, gels were incubated with 2.5% Triton X-100. Incubation was then performed for 18 h at 37°C in buffer containing 50 mM TrisHCl, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5. Gels were stained with Coomassie blue G-250 in 30% methanol, 10% acetic acid for 1 h and destained in the same solution without the dye. Gelatinase-containing bands were visualized as clear zones against a dark background using using an Agfa Duoscan 1200 in the transillumination mode. Pictures were processed using the Adobe photoshop software (Adobe).

HCAP18/LL-37 detection in neutrophil extracts by immunoblotting

Neutrophils were prepared by routine procedures from blood of healthy human donors. The cells (1×10^7 cells ml^{-1}) were disrupted by freeze-thawing and 5 μl of the supernatants were incubated with 5 μl of conditioned medium from elastase-producing *P. aeruginosa*, elastase-deficient *P. aeruginosa* or purified elastase (0.5 μg), TH was used as control. Incubation was performed at 37°C for various periods of time (see figure legend). The material was loaded onto 16.5% precast polyacrylamide gels and proteins/peptides were transferred to PVDF membranes (Immobilon, Millipore). Membranes were incubated for 1 h with rabbit polyclonal LL-37 antibodies (1:4500) (Innovagen AB, Lund), washed, and subsequently incubated (1 h) with HRP-conjugated secondary antibodies (1:1000). HCAP18/LL-37 was visualized using the ECL system (Boehringer).

Blotting assay for LL-37 binding

This was performed essentially as previously described (Schmidtchen *et al*. 2001b). LL-37 was applied onto nitrocellulose membranes (Hybond, Amersham). Membranes were blocked (PBS, pH 7.4, 0.25% Tween 20, 3% bovine serum albumin) for 1 h and incubated with radiolabelled DS (~10 μg ml^{-1}) for 1 h in the same buffer. Unlabelled polysaccharides (2 mg ml^{-1}) were added for competition of binding. The membranes were washed (3 × 10 min) (PBS, pH 7.4, 0.25% Tween 20). Bas 2000 radioimaging system (Fuji) was used for visualization of radioactivity.

Mass spectrometry

Analysis of LL-37 fragments was performed by electrospray ionisation mass spectrometry on a Q-tof (Micromass, UK) using Masslynx software (performed by Innovagen AB, Lund).

Bactericidal assays

For antimicrobial assays *S. pyogenes*, *E. faecalis,* or *P. aeruginosa* were grown to mid-log phase in TH-medium. Bacteria were washed and diluted in 10 mM Tris-HCl, pH 7.5, containing 5 mM glucose. 50 μl of bacteria (2×10^6 colony-forming units ml^{-1}) were incubated with intact or degraded LL-37, at the indicated concentrations (see figure legends). Incubations were carried out at 37°C for 2 h (*S. pyogenes*, *E. faecalis*) or 4 h (*P. aeruginosa*). To quantify the bactericidal activity, serial dilutions of the incubation mixture were plated on TH agar, incubated at 37°C overnight, and the number of colony-forming units were determined. In one experiment, the agar plates were placed on a light-board, photographed, and pictures further processed using Photoshop software (Adobe).

Bacterial growth assay

Pseudomonas aeruginosa were grown to stationary phase (OD ~ 1) in TH-medium. Then 200 μl of 10% sterile wound fluid (in PBS) was inoculated with 10 μl of the bacterial suspension in 96-well plates (Falcon). After an incubation period of 5 h at 37°C, LL-37 was added and absorbance measured at 490 nm (using a Bio-Rad 550 microplate reader) after various periods of time. The absorbance value at T = 5 h

(addition of LL-37) was subtracted from the thereafter attained values (see figure legends for further details).

Fluorescence microscopy

Bacteria (5×10^6 ml^{-1}) were incubated for 5 min on ice together with either intact Texas red-labelled LL-37 (10 μg ml^{-1}) or with Texas red-labelled LL-37 that had been subjected to prior treatment with *P. aeruginosa* elastase (5 ng of elastase μg^{-1} LL37 for 4 h at 37°C). Complete degradation of the peptide was verified by electrophoresis of the material on 16.5% Tris-tricine gels followed by UV-transillumination. After washing twice with ice-cold PBS, the bacteria were mounted on slides for immediate visual inspection and recording of images. This was performed using a Nicon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, a Plan Apochromat 60X objective, and a high N.A. oil-condenser.

FACS analysis

Flow cytometry measurements were performed using a FACSCalibur flow cytometer (Becton-Dickinson) equipped with a 15 mW argon laser tuned at 488 nm. The bacterial population was selected by gating with appropriate settings of forward scatter (FSC) and sideward scatter (SSC). The FL3 fluorescence channel ($\lambda_{em} = 585$ nm) was used to record the emitted fluorescence of Texas red. All experiments were performed at least three times using different preparations of bacteria and the results were analysed using the software CellQuest (Becton Dickinson).

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