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Processing of Seminal Plasma hCAP-18 to ALL-38 by Gastricsin

A NOVEL MECHANISM OF GENERATING ANTIMICROBIAL PEPTIDES IN VAGINA*

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The human cathelicidin, hCAP-18, is expressed both in neutrophils and in epithelial cells. hCAP-18 is processed to the antimicrobial peptide LL-37 by proteinase 3 in neutrophils. hCAP-18 is highly expressed in the epididymis with a subsequent high concentration in seminal plasma where the protein is present in its unprocessed and antimicrobially inactive form. We report here that hCAP-18 in seminal plasma is processed to generate a 38-amino acid antimicrobial peptide ALL-38 by the prostate-derived protease gastricsin when incubated at a pH corresponding to the vaginal pH. In accordance with this, seminal plasma derived hCAP-18 was found in its processed form in the vagina following sexual intercourse. The antimicrobial activity of ALL-38 against a variety of microorganisms tested is equal to that of LL-37. This enzymatic activation of a proantimicrobial substance in seminal plasma following exposure to the vaginal milieu represents a novel mechanism to prevent infection following sexual intercourse.

Antimicrobial peptides are important effector molecules of the innate immune system from insects to humans (1, 2). The peptides are active against a broad spectrum of Gram-positive and Gram-negative bacteria as well as some fungi and enveloped viruses. These peptides may play a role in the regulation of the normal microflora (3). In mammals, the antimicrobial peptides are of major importance for the antimicrobial efficacy of professional phagocytes such as neutrophils and macrophages, but the peptides are also expressed in epithelial cells (4). In mammals, there are two large families of antimicrobial peptides, defensins and cathelicidins. Defensins are divided into the α-defensins found in neutrophils, macrophages, and Paneth cells in the small intestine, and the β-defensins, which are found widespread in epithelial cells. Cathelicidins are found mainly in neutrophils (5). Members of this protein family share a highly conserved N terminus of 12 kDa, named cathelin after a protein isolated from porcine neutrophils (6).

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Antimicrobial peptides are synthesized as preproproteins and (with the exception of defensins in neutrophils) stored as inactive proproteins (7). In order to become biologically active, the peptides must be liberated from the proproteins by proteolytic cleavage.

The proteolytic generation of antimicrobial peptides is very important for the clearance of bacteria at sites of infection. In mice, α-defensins from Paneth cells in the small intestine are generated by matrilysin-mediated cleavage of prodefensins, and matrilysin knock-out mice have increased susceptibility to intestinal infections (8). Inhibition of activation of the porcine neutrophil cathelicidins by elastase impairs clearance of bacteria from wounds *in vivo* (9).

The only human cathelicidin, hCAP-18, is a major protein of the specific granules of human neutrophils (10). It is processed to the antimicrobial peptide LL-37 by extracellular cleavage by proteinase 3 from azurophil granules following exocytosis (11). LL-37 has broad spectrum antimicrobial activity toward Gram-negative and Gram-positive bacteria (12, 13). Furthermore, LL-37 binds and neutralizes the effects of lipopolysaccharide (14) and is a chemoattractant toward neutrophils, monocytes, and T-lymphocytes (15).

hCAP-18 is also present in epithelial cells (*i.e.* in various squamous epithelia (16), in the lungs (17, 18), in sweat glands (19), in salivary glands (20), and in keratinocytes during inflammatory disorders (21)). The strongest epithelial expression of hCAP-18 is found in the epididymis with a subsequent high concentration in seminal plasma (22, 23).

How the epithelium-derived hCAP-18 is processed to generate an active antimicrobial peptide is not known. Because of the high concentration of hCAP-18 in seminal plasma, we chose this as a model to study the processing and antimicrobial activation of hCAP-18 expressed in epithelial cells.

EXPERIMENTAL PROCEDURES

Materials

Vaginal fluid and freshly ejaculated semen was collected from healthy volunteers at an outpatient fertility clinic. The semen was allowed to liquefy for 1 h at room temperature followed by centrifugation at 800 $\times$ *g* for 5 min, and the supernatant was collected. Both the seminal plasma and the vaginal fluid were then stored at −20 °C until further use. After thawing, the seminal plasma and vaginal fluid were used immediately for cleavage experiments. Semen was installed in the vagina of women undergoing insemination and collected after 4 h. Postcoital vaginal fluid was collected 10 h following sexual intercourse and stored at −20 °C until further analysis.

Polyclonal anti-hCAP-18 antibodies were previously described (24). A monoclonal antibody against LL-37 was generated by immunization of mice with glutaraldehyde-cross-linked synthetic LL-37 using conven-

tional hybridoma technology.[1] Polyclonal rabbit antibodies against progastricsin (pepsinogen C) were purchased from U.S. Biological (Swampscott, MA).

Synthetic LL-37 and ALL-38 were purchased from Schafer-N (Copenhagen, Denmark). The peptides were synthesized using the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) strategy, purified by reverse phase chromatography, and analyzed by HPLC coupled to a Shimadzu LCMS-QP8000 spectrometer. The purity of the peptides was greater than 95%. Gastricsin, purified as described, was generously provided by Pal B. Szecsi (25).

Cleavage Experiments

The pH of samples of seminal plasma was adjusted to pH 4 by the addition of 0.5 M sodium acetate and incubated at 37 °C for 6 h. Following incubation, the pH was neutralized by the addition of Tris buffer, and the samples were boiled in Laemmli sample buffer.

SDS-PAGE and Immunoblotting

SDS-PAGE (26) and immunoblottting (27) were performed with Mini-Protean 3 cells and Mini Trans-Blot Electrophoretic Transfer cells according to the instructions given by the manufacturer (Bio-Rad). For immunoblotting, the polyvinylidene difluoride (PVDF)[2] membranes (Millipore Corp., Bedford, MA) were blocked for 1 h with 5% skimmed milk in phosphate-buffered saline after the transfer of proteins from the 14% SDS-polyacrylamide gels. For detection of hCAP-18 or fragments derived thereof, the PVDF membranes were incubated overnight with primary antibodies. The following day, the membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and visualized by diaminobenzidine/metal concentrate and stable substrate buffer (Pierce).

Purification and Identification of hCAP-18 Fragments Generated by Cleavage in Seminal Plasma

Fragments of hCAP-18, generated by cleavage of hCAP-18 in seminal plasma at pH 4, were affinity-purified on an anti-hCAP-18 antibody column.

To identify the cathelin part, the eluted material was dialyzed against 25 mM Tris (pH 8.5) and subjected to anion exchange chromatography on a MonoQ column using Äkta-FPLC (Amersham Biosciences). Bound material was eluted with a gradient of NaCl from 25 mM to 1 M in 50 mM Tris, pH 8.0. One peak containing proteins of ~14 kDa was eluted at 0.2 M NaCl. The protein was repurified and desalted by reverse phase HPLC employing a Vydac C4 column (2.1 × 150 mm) equilibrated with 10% solvent B and eluted with a 1%/min gradient from solvent A to solvent B (solvent A: 0.1% trifluoroacetic acid; solvent B: 0.1% trifluoroacetic acid in acetonitrile). The purified protein was reduced and derivatized with iodoacetamide, as described by Matsudaira (28), followed by HPLC purification as described above. The derivatized cathelin was dissolved in 50 μl of 50 mM sodium phosphate (pH 8.0) and digested with 0.5 μg of endoproteinase Asp-N (Roche Molecular Biochemicals) overnight at 37 °C. Then 50 μl of 5% formic acid was added, and the reaction mixture was loaded onto a C18 ZipTip (Millipore), washed with 0.5% formic acid, and eluted with 10 μl of 0.5% formic acid in 50% acetonitrile. An aliquot of 2 μl of the purified peptide mixture was introduced into a Q-Tof-2 tandem mass spectrometer (Micromass, Manchester, UK) using the nanospray interface and analyzed in MS mode as well as in MSMS mode for selected fragments to confirm their structure.

Amino Acid Sequence Analysis

Amino acid sequence was analyzed on the PVDF-blotted protein in a 494 A Procise Protein Sequencer (PerkinElmer Life Sciences) using the blot cartridge and PVDF cycles. All reagents and solvents were supplied by PerkinElmer Life Sciences.

Immunoprecipitation

Antibodies against gastricsin and normal rabbit immunoglobulins were incubated with Protein A-Sepharose (Amersham Biosciences) for 30 min at room temperature in phosphate-buffered saline (pH 7) with 0.5 M NaCl. The Sepharose particles were subsequently washed seven times in phosphate-buffered saline with 0.5 M NaCl to remove unbound antibodies followed by incubation with seminal plasma at 4 °C for 4 h. 0.5 M NaCl were added to the seminal plasma before immunoprecipitation to prevent nonspecific absorption to the Sepharose particles. The Sepharose particles were pelleted by centrifugation. The supernatants were aspirated and immediately used for cleavage experiments.

Purification of hCAP-18 from Seminal Plasma and Neutrophils for Cleavage Experiments

Seminal plasma was applied to an affinity chromatography column with anti-hCAP-18 antibodies immobilized on CNBr-activated Sepharose (Amersham Biosciences) as described by the manufacturer. The column was washed extensively, and the bound protein was eluted with 0.2 M glycine HCl (pH 2.5).

hCAP-18 was purified from specific granules of human neutrophils by affinity chromatography and by cation exchange chromatography as previously described (11).

Antibacterial Activity of ALL-38 and LL-37

Growth Inhibition—The bacterial strains of *Escherichia coli* (ATCC 25922 and strain 1655 (genome-sequenced K12 strain)); *Staphylococcus aureus* (ATCC 25923); *Bacillus megaterium* (Bm11), and *Pseudomonas aeruginosa* PAO1 were tested for purity on trypticase soy agar (Oxoid CM131) and inoculated in M9 salts (29) with glucose and casamino acids (M9GC) overnight at 37 °C. LL-37 and ALL-38 were dissolved in M9CG at 100 μg/ml and sterile-filtered, and further 10-fold dilutions were prepared in sterile M9CG. The peptide containing M9GC was pipetted into 96-microwell plates in volumes of 200 μl. All concentrations were tested in duplicate. The plate included M9GC with no peptide as growth control and noninoculated M9GC for check of sterile conditions. Experiments were conducted with M9GC adjusted to pH 7 and M9GC adjusted to pH 5 (using HCl) to determine whether increased antibacterial activity was observed at the lower pH.

The wells were inoculated with 20 μl of a 10-fold dilution of *Bacillus* and *Staphylococcus* and 20 μl of a 100-fold dilution of the two *E. coli* strains and of *P. aeruginosa*. This corresponded to the addition of 2×10^5 cfu to each well. Absorbance at 450 nm was read, and the plate was incubated at 37 °C. Absorbance was read after 1, 2, 3, 6, 8, and 10 days. At day 10, 10-fold dilution series were prepared from all wells with peptides where bacterial growth had not occurred (*S. aureus* and *B. megaterium*). Colony counts were made by surface plating on trypticase soy agar.

It has previously been noted that the composition of the growth medium is important when testing the antibacterial activity of LL-37 (12). Accordingly, we used a defined medium M9GC. In this medium, we repeatedly found a growth delay of *S. aureus*, which was not found in nutrient-rich media like brain heart infusion. This could be due to the autoinhibition, which has been described for other Gram-positive bacteria (30, 31).

Bactericidal Activity against Log Phase Cells—E. coli MG1655 was precultured in M9GC and inoculated at ~100 cfu/ml in M9GC and incubated at 37 °C with aeration. When A_{450} nm reached 0.1, the culture was diluted 1000-fold in medium and mixed (400-μl volume) with sterile-filtered solutions of LL-37 and ALL-38 (125-μl volumes) dissolved in distilled water. Concentrations of peptides tested were 0, 3, 6, 12, 25, 50, and 100 μg/ml. The bacteria-peptide mixtures were incubated at 37 °C, and samples were taken immediately after mixing for colony counts and after 2, 6, and 24 h.

Bactericidal Activity against Stationary Phase Cells—S. aureus was cultured in brain heart infusion (Oxoid CM225), and *E. coli* was cultured in M9GC overnight at 37 °C. The cultures were harvested at 3,500 × *g* for 3 min and resuspended in either PIPES (pH 7; Sigma catalog no. P6757) or HOMOPIPES (pH 4). Absorbance at 450 nm was adjusted to 0.2. The bacterial suspensions were mixed (equal volumes) with peptide ALL-38, resulting in final concentrations of 50 or 5 or 0.5 μg/ml at both pH values. Stock solutions of peptide were prepared in either PIPES or HOMOPIPES. Bacterial levels were determined by surface plating 0, 3, and 24 h after mixing.

RESULTS

Cleavage of hCAP-18 in Seminal Plasma at Low pH—hCAP-18 is present in seminal plasma, appearing as a double band around 18 kDa as observed by immunoblotting (22). Incubation of seminal plasma at neutral pH for 6 h at 37 °C did not change the observed molecular forms of hCAP-18 (Fig. 1, *A*

[1] G. S. Tjabringa, J. Aarbiou, D. K. Ninaber, J.-W. Drijfhout, O. E. Sørensen, N. Borregaard, K. F. Rabe, and P. S. Hiemstra, submitted for publication.

[2] The abbreviations used are: PVDF, polyvinylidene difluoride; PIPES, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid; HOMOPIPES, homopiperazin-*N*,*N*′-bis-2-(ethansulfonsyre); HPLC, high pressure liquid chromatography; MS, mass spectrometry; MSMS, tandem MS; cfu, colony-forming units.

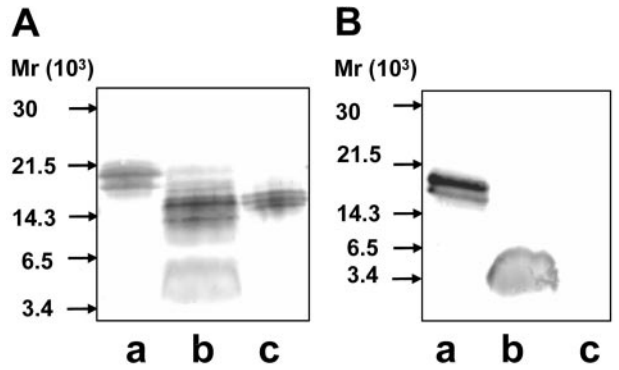

FIG. 1. **Immunoblots of hCAP-18 in seminal plasma and neutrophils.** Samples were subjected to SDS-PAGE and immunoblotting was performed with either polyclonal anti-hCAP-18 antibodies (*A*) or monoclonal anti-LL-37 antibody (*B*). *Lane a*, seminal plasma (at neutral pH). *Lane b*, seminal plasma following incubation at pH 4. *Lane c*, 14-kDa fragments generated in seminal plasma following incubation at pH 4, purified by affinity chromatography on an anti-hCAP-18 antibody column and anion exchange chromatography.

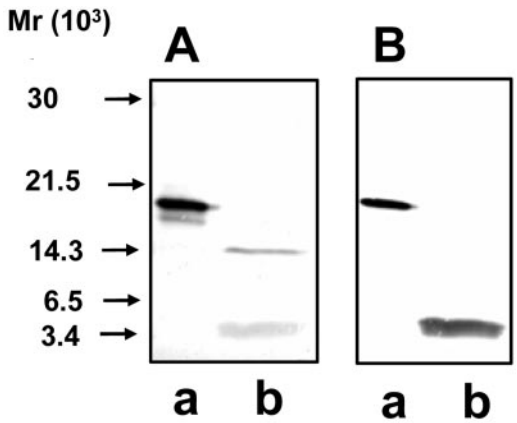

FIG. 2. **Cleavage of neutrophil-derived hCAP-18 in hCAP-18-depleted seminal plasma.** Endogenous hCAP-18 was depleted from seminal plasma by applying seminal plasma on an anti-hCAP-18 antibody column. The hCAP-18-depleted seminal plasma was then incubated with neutrophil-derived hCAP-18 for 6 h at 37 °C at neutral pH (*lane a*) or at pH 4 (*lane b*). The samples were subjected to SDS-PAGE, and immunoblotting was performed with either polyclonal anti hCAP-18 antibodies (*A*) or monoclonal anti LL-37 antibody (*B*).

and *B*, *lane a*). However, following incubation at pH 4, bands were observed around 14 kDa together with a low molecular weight band at 5–6 kDa when immonoblotting was performed with polyclonal anti-hCAP-18 antibodies (Fig. 1*A*, *lane b*), but only the 5–6-kDa band was observed when blotting was done with the monoclonal anti-LL-37 antibody (Fig. 1*B*, *lane b*). Thus, cleavage of endogenous hCAP-18 in seminal plasma at low pH generates a fragment at the size of LL-37, which reacts with the monoclonal anti-LL-37 antibody. The bands at ~14 kDa detected with the polyclonal anti-hCAP-18 antibodies were not detected by the monoclonal anti-LL-37 antibody. These fragments represent the cathelin part of hCAP-18 as further validated below. This corresponds to the cleavage of hCAP-18 found in exocytosed material from neutrophils, where LL-37 is cleaved from the cathelin part (11).

The double bands of hCAP-18 observed previously in seminal plasma (22, 32) must be due to either posttranslational modifications or to proteolytic processing occurring during sampling of the ejaculate. To exclude an artifactual proteolysis occurring during sampling, endogenous hCAP-18 was removed, and the ability of seminal plasma to process purified hCAP-18 from neutrophil-specific granules was examined. Fig. 2 shows that purified hCAP-18 is processed to generate a low molecular weight band at low pH, which is recognized by the monoclonal anti-LL-37 antibody (Fig. 2*B*, *lane b*) and an additional single band of 14 kDa, which can be observed when immunoblotting is performed with the polyclonal anti-hCAP-18 antibodies (Fig. 2*A*, *lane b*). This argues that the endogenous epididymal-derived hCAP-18 and exogenous neutrophil-derived hCAP-18 are processed in a similar way in seminal plasma and that the double band appearance of epididymal-derived hCAP-18 is due to a possible posttranslational modification and not to proteolysis occurring during sampling.

Identification of hCAP-18 Fragments Generated in Seminal Plasma at Low pH—To further identify the hCAP-18 fragments generated in seminal plasma at pH 4, these fragments were affinity-purified using an anti-hCAP-18 antibody column.

An aliquot of the eluate from the antibody column was concentrated by precipitation with trichloroacetic acid and was subjected to SDS-PAGE and blotted to a PVDF membrane. The low molecular mass fragment of 5–6 kDa was analyzed by N-terminal amino acid sequencing of the first 10 residues. These were identified as ALLGDFFRKS, consistent with the 38-amino acid peptide ALL-38.

To purify the peptides of 14 kDa seen by immunoblotting with polyclonal anti-hCAP-18 antibodies, the remains of the eluate from the antibody column were subjected to anion exchange chromatography. The 14-kDa peptides eluted at ~0.2 M NaCl. As expected, these peptides were not recognized by the monoclonal anti LL-37 antibody but only by the polyclonal anti-hCAP-18 antibodies (Fig. 1, *A* and *B*, *lane c*). The N terminus of hCAP-18 (and of the cathelin part) is blocked for protein sequence analysis (33). In order to further identify the purified 14-kDa peptides, fragments hereof were generated by cleavage with endoproteinase Asp-N and identified by mass spectrometry. All of the generated fragments corresponded to segments in the cathelin part of hCAP-18 (Fig. 3). The most C-terminal fragment identified was the fragment 98–102 DNKRF (Table I), which is the expected C terminus of the cathelin part of hCAP-18 if this is cleaved to liberate ALL-38.

The immunoblotting with the monoclonal anti-LL-37 antibody and the identity of purified fragments generated in seminal plasma demonstrated that hCAP-18 was cleaved between the cathelin part of the molecule and its C-terminal peptide ALL-38 in seminal plasma.

Antimicrobial Activity of LL-37 and ALL-38—To verify the assumed antimicrobial activity of ALL-38, both synthetic LL-37 and ALL-38 were generated. The antimicrobial activity of LL-37 and ALL-38 was tested against strains of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. megaterium* (Fig. 4), where no difference was found between the two peptides. Both *E. coli* strains were inhibited by 100 μg/ml for 2 days, after which normal growth occurred. The growth of *P. aeruginosa* was not affected by the presence of the peptides (data not shown). The Gram-positive strains were more sensitive, being inhibited by 100 and 10 μg/ml for 10 days.

At day 10, 10-fold dilution series were made from samples with peptides where no growth had occurred (*B. megaterium* and *S. aureus*) (*i.e.* from wells where the absorbance remained as the sterile control). Colony counts were made by surface plating on trypticase soy agar. No growth was detected from wells inoculated with *B. megaterium*, whereas colonies were detected from *S. aureus*-containing wells where growth had not occurred. These findings indicate that the peptides had a bactericidal effect toward *B. megaterium* and a bacteriostatic effect toward the staphylococci. The same result was found for both LL-37 and ALL-38. The results also indicate that the peptides were stable over time, since the bacteriostatic effect was retained for more than a week.

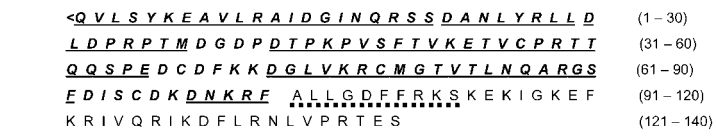

FIG. 3. **Identification of the fragments generated by cleavage of hCAP-18 in seminal plasma.** The amino acid sequence of hCAP-18 is shown (<*Q* denotes pyroglutamic acid). The residues are *numbered* on the *right*. The sequence of the cathelin part is shown in *boldface italic type*. Fragments of the 14-kDa proteins were generated by cleavage with endoproteinase Asp-N, identified by mass spectrometry (see also Table I). The identified fragments are *underlined* with a *solid line*. The structure of the C-terminal fragment (DNKRF) of the cathelin part was furthermore supported by MSMS spectrum providing sequence information. The first 10 amino acid residues of the C-terminal antimicrobial peptide were identified by amino acid sequence analysis. These residues are *underlined* with a *dotted line*.

TABLE I

Analysis of fragments produced by endoproteinase Asp-N cleavage of the 14-kDa fragments of hCAP-18 generated in seminal plasma at low pH

The 14-kDa fragments of hCAP-18 purified from seminal plasma were reduced, and the Cys residues were derivatized with iodoacetamide. The protein was then digested with endoproteinase Asp-N, and the resulting mixture of fragments was analyzed by mass spectrometry.

Fragment	Molecular mass	
	Experimental	Theoretical[a]
	Da	
1–21[b]	2329.26	2329.24[c]
22–29	976.54	976.53
30–37[b]	943.45	943.44
38–39	ND[d]	190.06
40–41	ND	231.09
42–65	2731.38	2731.35
66–67	ND	293.07
68–71	ND	536.29
72–91	2209.14	2209.11
92–95	ND	493.18
96–97	ND	261.13
98–102	678.37[e]	678.34

[a] Including carboxamide derivative of Cys residues. All figures are given as monoisotopic values.
[b] Resulting from incomplete cleavage.
[c] Assuming the N-terminal residue to be pyroglutamate.
[d] ND, not determined.
[e] The identification was supported by MSMS-spectrum providing sequence information.

Exponentially growing *E. coli* cells were then exposed to the peptides in different concentrations. Initial concentrations of cells were approximately 10^6 cfu/ml. Immediately after mixing, the peptides caused a reduction in culturable count of approximately 1 log unit (Table II). At the sampling after 2 h, both LL-37 and ALL-38 caused a dramatic reduction of viable cells to or below 10^4 cfu/ml at 12–100 μg/ml. After 6 h, cell densities in the three highest concentrations were approximately 10^2 to 10^3 cfu/ml. Bacterial densities remained low in the highest concentrations of the two peptides 24 h after mixing, whereas growth resumed in the lower concentrations. Again, the antibacterial activity of ALL-38 was equal to that of LL-37.

Since no difference was seen between the two peptides, only ALL-38 was used to test bacteriolytic activity on nongrowing cell suspensions. No bacteriolytic activity was seen when cells suspended in buffers were exposed to the peptide, independent of pH (Table III). Overall, our findings indicate that the peptides primarily are active against growing bacterial cells.

Identification of the Protease Responsible for Cleavage of hCAP-18 in Seminal Plasma—To identify the protease responsible for the generation of the antimicrobial peptide ALL-38, protease inhibitors were added to seminal plasma before incubation at pH 4. The cleavage of hCAP-18 was not inhibited by phenylmethylsulfonyl fluoride or aprotinin (general inhibitors of serine proteases), EDTA (inhibitor of metalloproteases), chymostatin (chymotrypsin-like protease inhibitor), or E-64 (cysteine protease inhibitor) (Fig. 5, *lanes b–f*). The cleavage of hCAP-18 was, however, totally abolished by pepstatin A (an aspartic protease inhibitor) (Fig. 5, *lane g*). Low pH activates the proform of the aspartic protease gastricsin (pepsin C) in seminal plasma (34), and the addition of the specific pepsin inhibitor, pepsinostreptin, totally abolished the cleavage of hCAP-18 (Fig. 5, *lane h*).

To further validate that gastricsin was responsible for the cleavage of hCAP-18, gastricsin was immunoprecipitated from seminal plasma. Following precipitation with preimmune rabbit antibodies, cleavage was still observed at low pH (Fig. 6, *lane b*). However, following immunoprecipitation with anti-gastricsin antibodies, cleavage of hCAP-18 was abolished (Fig. 6, *lane a*). The antibodies used for immunoprecipitation of gastricsin reacted with purified gastricsin (data not shown).

Cleavage Experiments with Purified Gastricsin—To further verify the gastricsin-mediated cleavage of hCAP-18 in seminal plasma, hCAP-18, purified from both neutrophils and seminal plasma, was incubated with gastricsin at pH 4. Gastricsin cleaved hCAP-18 from both sources (Fig. 7, *A* and *B*, *lanes b* and *d*). The cleavage of seminal plasma-derived hCAP-18 gave rise to two bands at ~14 kDa, and the neutrophil-derived hCAP-18 gave rise to one band at 14 kDa as visualized by immunoblotting with polyclonal antibodies, in addition to the 5–6-kDa band. This low molecular weight band and the unprocessed hCAP 18 were detected by the monoclonal anti-LL-37 antibody, whereas the bands at 14 kDa were not. When hCAP-18 was incubated with gastricsin at neutral pH, no cleavage was observed (Fig. 7*A*, *lane e*). The cleavage of hCAP-18 by gastricsin thus resembles that in seminal plasma at low pH.

In Vivo Processing of hCAP-18 following Sexual Intercourse—To validate that the processing of hCAP-18 takes place *in vivo*, samples of vaginal fluid, seminal plasma collected from vagina 4 h after installation, and a postcoital sample were analyzed. Very little hCAP-18 is present in normal vaginal fluid (Fig. 8, *A* and *B*, *lane b*). When samples of seminal plasma inseminated in vagina were collected after 4 h, the pH of the samples was neutral, and no processing of hCAP-18 had occurred (data not shown). A vaginal sample obtained 10 h after sexual intercourse had a pH value below 5. Immunoblotting of this with polyclonal anti-hCAP-18 antibodies demonstrated both a double band at 18 kDa corresponding to unprocessed hCAP-18 and 14 kDa corresponding to the cathelin part of processed hCAP-18 (Fig. 8, *A* and *B*, *lane c*). When immunoblotting was performed with the monoclonal anti-LL-37 antibody, only the double band at 18 kDa (Fig. 8*B*, *lane c*) was visualized, not the 14 kDa in agreement with the interpretation that this latter is the cathelin part of hCAP-18. The double bands at both 18 and 14 kDa observed in the postcoital vaginal sample, furthermore, demonstrate that the observed hCAP-18 is derived from seminal plasma, since processed hCAP-18 from neutrophils would only have one band at 18 kDa and one at 14 kDa. We were, however, not able to detect any band at 5–6 kDa in the postcoital sample by immunoblotting either with the monoclonal or with the polyclonal antibody.

To rule out that the observed *in vivo* processing of hCAP-18 was due to a vaginal fluid-derived protease, vaginal fluid was then mixed with seminal plasma in a ratio of 10:1. Because of the buffer capacity of the seminal plasma, the pH remained neutral, and no cleavage of hCAP-18 was observed following prolonged incubation (Fig. 8, *A* and *B*, *lane d*). When the vaginal fluid was incubated with seminal plasma and the pH was adjusted to 4, cleavage was observed similar to the cleavage of

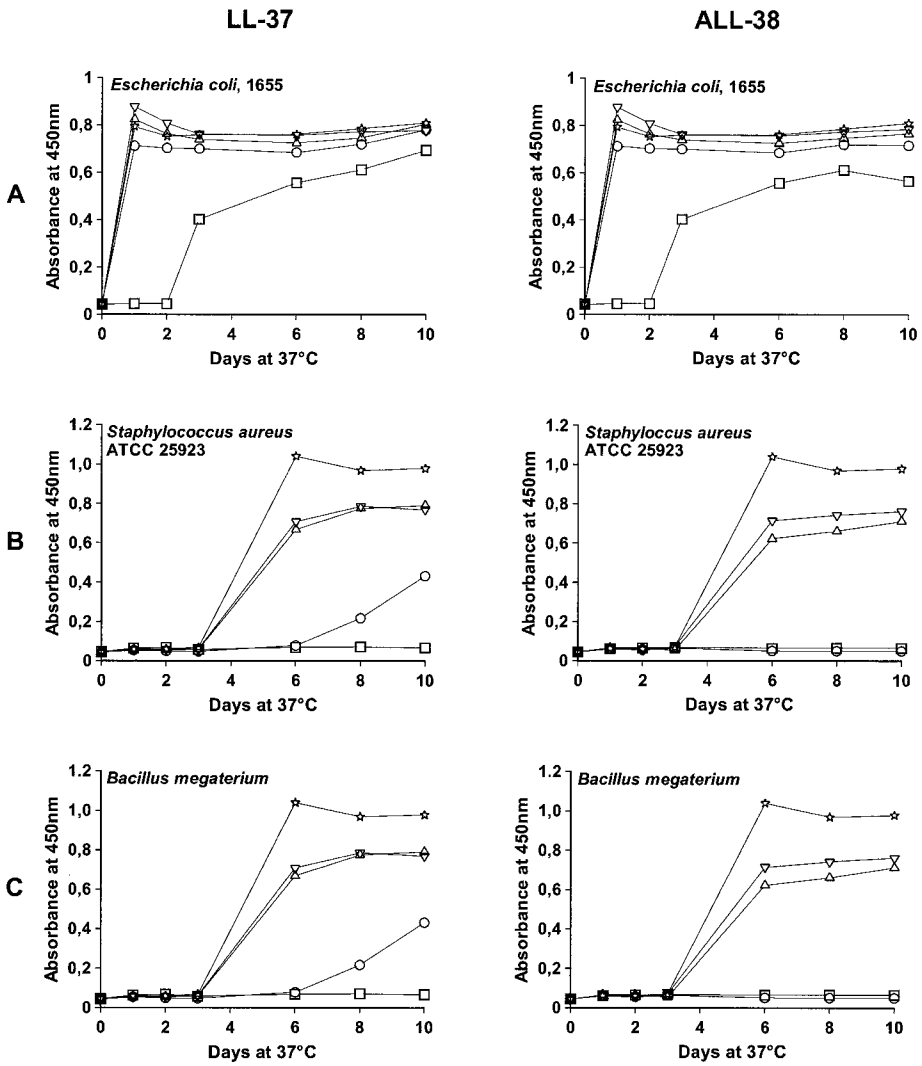

FIG. 4. **Antimicrobial activity of ALL-38 and LL-37.** The bacterial strains *E. coli* ATCC 1655 (*A*), *S. aureus* ATCC 25923 (*B*), and *B. megaterium* Bm11 (*C*) were grown in the absence and presence of different concentrations of LL-37 and ALL-38 in microwell plates. □, 100 μg/ml; ○, 10 μg/ml; △, 1 μg/ml; ▽, 0.1 μg/ml; ☆, 0 μg/ml (control). The absorbance was measured at 450 nm at days 0, 2, 4, 6, and 10 to monitor bacterial growth.

TABLE II

Bacteriolytic effect of LL-37 and ALL-38 on growing cells of E. coli in M9GC at 37°C

The log phase bacteria were exposed to peptides for up to 24 h. Bacterial levels were determined by surface plating 0, 3, and 24 h after mixing.

Peptide	Time	Bacterial levels						
		0[a]	3.2	6.3	12.5	25	50	100
	h				*cfu/ml*			
LL-37	0	8.0×10^5	1.1×10^6	1.2×10^6	7.0×10^5	4.5×10^5	2.9×10^5	1.0×10^5
	3	7.2×10^6	1.2×10^7	3.4×10^6	2×10^4	$<10^4$	$<10^4$	$<10^4$
	6	3.0×10^8	3.4×10^8	1.8×10^8	3×10^5	1×10^2	2.8×10^3	1×10^3
	24	6.0×10^8	1.3×10^9	1.0×10^9	1.0×10^9	5.7×10^4	$<10^2$	5×10^2
ALL-38	0	1.2×10^6	1.3×10^6	1.2×10^6	1.2×10^6	3.6×10^5	4.2×10^5	5×10^4
	3	8.0×10^6	1.1×10^7	1.2×10^7	1×10^5	$<10^4$	$<10^4$	$<10^4$
	6	3.8×10^8	4.5×10^8	3.7×10^8	6.5×10^5	1×10^2	$<10^2$	1.0×10^2
	24	7.0×10^8	9.0×10^8	1.2×10^9	1.2×10^9	3.3×10^5	$<10^2$	6.0×10^2

[a] Concentration of peptide in μg/ml.

hCAP-18 in seminal plasma at low pH (Fig. 8, *A* and *B*, *lanes e* and *f*). The cleavage of hCAP-18 in seminal plasma mixed with vaginal fluid at low pH was also inhibited by pepsinostreptin as in "pure" seminal plasma at low pH, demonstrating that also in the presence of vaginal fluid, hCAP-18 was processed by gastricsin at low pH (Fig. 8, *A* and *B*, *lanes g* and *h*). It is, furthermore, noteworthy that the mature ALL-38 was not degraded in the presence of vaginal fluid at low pH.

These findings are in accordance with previous *in vivo* studies of the activation of gastricsin in the vagina, where gastricsin was activated only several hours after either sexual intercourse or insemination in the vagina and paralleled a drop in the vaginal pH to acidic levels (35). The observed postcoital processing of hCAP-18 *in vivo* was therefore similar to that observed in seminal plasma at low pH.

DISCUSSION

We have found that the promicrobicidal protein, hCAP-18, which is present in seminal plasma at high concentrations (22), is processed by gastricsin to generate the antimicrobial peptide ALL-38 at a low pH similar to the pH in the vagina. A similar processing was found in the vagina after sexual intercourse.

TABLE III
Bacteriolytic effect of ALL-38 on nongrowing cells of S. aureus and E. coli

The stationary phase bacteria were suspended in buffers at pH 7 (PIPES) and pH 4 (HOMOPIPES) and exposed to ALL-38 for up to 24 h. Bacterial levels were determined by surface plating 0, 3, and 24 h after mixing. No bacteriolytic activity was observed at either pH.

Organism	Time	pH	Bacterial levels	
			No addition	50 μg/ml
	h		*cfu/ml*	
S. aureus	0	7	2.0×10^7	
		4	1.4×10^7	
	3	7	3.0×10^7	4.0×10^7
		4	1.0×10^7	5.0×10^6
	24	7	4.0×10^7	3.0×10^7
		4	3.0×10^5	2.5×10^5
E. coli 1655	0	7	1.6×10^7	
		4	3.6×10^7	
	3	7	2.5×10^7	3.2×10^7
		4	2.4×10^7	2.7×10^7
	24	7	3.5×10^7	2.4×10^7
		4	2.0×10^7	2.0×10^7

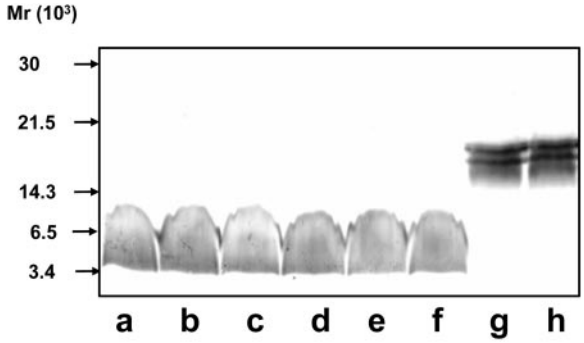

FIG. 5. **Effect of protease inhibitors on cleavage of hCAP-18 in seminal plasma.** Samples were subjected to SDS-PAGE followed by immunoblotting with monoclonal anti-LL-37 antibody. Following incubation at pH 4 (as found in the vagina) for 6 h, all of the hCAP-18 in seminal plasma was cleaved to generate ALL-38 (*lane a*). The addition of phenylmethylsulfonyl fluoride (*lane b*), aprotinin (*lane c*), EDTA (*lane d*), chymostatin (*lane e*), or E-64 (*lane f*) did not inhibit the cleavage of hCAP-18. The addition of pepstatin A (*lane g*) or pepsinostreptin (*lane h*) totally abolished the cleavage of hCAP-18 at pH 4.

Whereas we easily detected the holoprotein and the cathelin fragments of hCAP-18 in the postcoital sample, we were unable to detect the C-terminal antimicrobial domain. This is not likely due to proteolytic degradation of this peptide. Our data show that processing of hCAP-18 in vagina only takes place after several hours preceded by a drop in the pH. In the cleavage experiments with seminal plasma, where the pH instantaneously was adjusted to 4, we found no visible degradation of ALL-38 after 6 h although hCAP-18 in this setting was nearly completely processed. Furthermore, the proteolytic activity toward hCAP-18 was only found in seminal plasma, and the presence of vaginal fluid at low pH did not cause degradation of ALL-38. A likely explanation for our inability to recover the ALL-38 peptide could be that this very cationic and hydrophobic peptide binds to substances in the vagina (*e.g.* polyanions) that were not collected during the postcoital sampling.

It has previously been found that gastricsin degrades many seminal plasma proteins at low pH *in vitro* (36). This role of gastricsin was subsequently confirmed by *in vivo* studies (35), where it was demonstrated that the gastricsin in seminal plasma is activated at the low pH in the vagina 2–7 h postcoitus and that gastricsin activity is present more than 24 h thereafter (35). Thus, gastricsin-mediated cleavage of seminal plasma

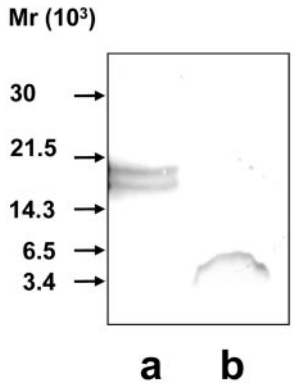

FIG. 6. **Immunoprecipitation of gastricsin from seminal plasma abolishes cleavage of hCAP-18.** Gastricsin was immunoprecipitated from seminal plasma, followed by incubation of the plasma at low pH. The samples were subjected to SDS-PAGE and immunoblotting performed with monoclonal anti-LL-37 antibody. In seminal plasma, no cleavage of hCAP-18 was observed after the removal of gastricsin by immunoprecipitation (*lane a*). When normal rabbit immunoglobulin (negative control) was used for immunoprecipitation, complete cleavage of hCAP-18 was observed (*lane b*).

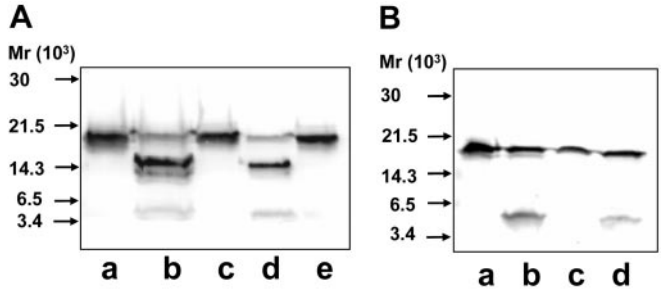

FIG. 7. **Cleavage of hCAP-18 with purified gastricsin.** hCAP-18 affinity-purified from seminal plasma and neutrophils were incubated with or without gastricsin at pH 4. The samples were subjected to SDS-PAGE and immunoblotting performed with polyclonal anti-hCAP-18 antibodies (*A*) or monoclonal anti-LL-37 antibody (*B*). Purified hCAP-18 from seminal plasma (*lane a*) or neutrophils (*lane c*) were not cleaved by incubation at pH 4. At pH 4, gastricsin cleaved hCAP-18 from seminal plasma (*lane b*) and neutrophils (*lane d*). No cleavage of hCAP-18 was observed following incubation of hCAP-18 with gastricsin at neutral pH (*lane e*).

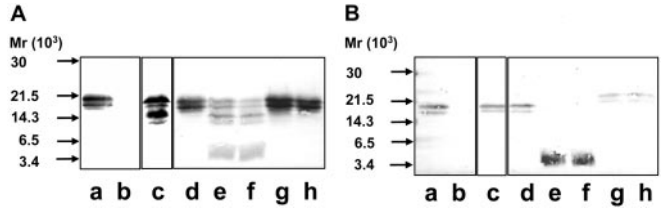

FIG. 8. **Immunoblots of hCAP-18 in seminal plasma, vaginal fluid, and postcoital vaginal sample.** Samples were subjected to SDS-PAGE and immunoblotting were performed with either polyclonal anti-hCAP-18 antibodies (*A*) or monoclonal anti-LL-37 antibody (*B*). *Lane a*, seminal plasma; *lane b*, vaginal fluid; *lane c*, vaginal sample collected 10 h after sexual intercourse; *lane d*, vaginal fluid and seminal plasma following incubation at 37 °C; *lane e*, vaginal fluid and seminal plasma following incubation at 37 °C at pH 4; *lane f*, seminal plasma following incubation at 37 °C and at pH 4; *lane g*, vaginal fluid and seminal plasma with the addition of pepsinostreptin following incubation at 37 °C at pH 4; *lane h*, seminal plasma with the addition of pepsinostreptin following incubation at 37 °C and pH 4.

proteins is a well documented *in vivo* phenomenon. In contrast to seminal plasma, vaginal fluid has a very low protein content (37), and no protease activity has been detected.

Following incubation of seminal plasma at pH 4 for 6 h, most of the hCAP-18 was cleaved by gastricsin into two parts: the cathelin part and ALL-38. The cleavage site between ALL-38

and the cathelin part was found both by determination of the N terminus of the C-terminal antimicrobial peptide and of the C terminus of the cathelin part. Both corresponded to a cleavage between the phenylalanyl and the alanyl residue, whereas in exocytosed material from neutrophils, cleavage is observed between the alanyl and leucyl residue (38, 39).

Thus, in seminal plasma, the cleavage of hCAP-18 leads to generation of ALL-38, in contrast to neutrophils, where cleavage of hCAP-18 leads to generation of LL-37. Peptides comprising the 33, 35, 37, and 39 C-terminal residues of hCAP-18 all possess antimicrobial activity (38). The 37-amino acid peptide (LL-37) was found to be the most potent of these hCAP-18-derived antimicrobial peptides (38). The antimicrobial activity of ALL-38 has not previously been determined. We found it to be equal to that of LL-37. Both LL-37 and ALL-38 were inhibitory (bacteriostatic or bacteriocidal) when tested on actively growing cells, and a killing effect was exerted against *Bacillus* and *E. coli*. In contrast, no antimicrobial effect was found on nongrowing bacterial cells suspended in buffer.

Other human promicrobicidal proteins are cleaved to generate antimicrobial peptides of different size (*e.g.* human β-defensin 1 and human α-defensin-5). Human β-defensin 1 has been purified from human urine with different N-terminal truncations (40). However, these peptides were not equally antimicrobially active (40), and how the different forms of human β-defensin 1 are generated from the proprotein is not known. Human α-defensin-5 has been isolated from ileal tissue in different forms, all of which are presumably generated by the same enzyme, trypsin (41).

The concentration of hCAP-18 in seminal plasma is around 85 μg/ml with a range between 40 and 140 μg/ml (22). This corresponds to a concentration of ALL-38 of 24 μg/ml with a range between 12 and 40 μg/ml. This is within the concentration range where we found antimicrobial activity of ALL-38.

Although processing of epithelium-derived hCAP-18 in seminal plasma and of neutrophil-derived hCAP-18 are mediated by different proteases, there are important similarities in these processes. In neutrophils, the expression and localization of hCAP-18 and the processing enzyme, proteinase 3, are separated although they are present in the same cells. Proteinase 3 is localized in azurophil granules (42), and hCAP-18 is localized in specific granules (10). hCAP-18 and proteinase 3 are localized together extracellularly following exocytosis, and only then is LL-37 generated (11). This strict control of generation of LL-37 is probably important because of the cytotoxic effects of this peptide (43). The hCAP-18 in seminal plasma is derived from epithelial cells in epididymis (22, 23), whereas gastricsin is derived from the prostatic gland and seminal vesicles (44). Thus, hCAP-18 and gastricsin only co-localize following ejaculation. Furthermore, significant processing requires activation of gastricsin by the low pH of vagina, which indicates that premature processing of the hCAP-18 stored in epididymis is prevented.

In summary, we found that the human cathelicidin, hCAP-18, is processed in seminal plasma to generate a novel antimicrobial peptide ALL-38 by gastricsin and that this process takes place following intercourse. The enzymatic activation of an antimicrobial substance in seminal plasma following exposure to the vaginal milieu represents a hitherto undiscovered mechanism to prevent infection following sexual intercourse.

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