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Research Article

New antimicrobial activity for the catecholamine release-inhibitory peptide from chromogranin A

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Abstract. Catestatin ($bCGA_{344-364}$), an endogenous peptide of bovine chromogranin A, was initially characterized for its effect on the inhibition of catecholamine release from chromaffin cells. Catestatin and its active domain ($bCGA_{344-358}$) were identified in chromaffin cells and in secretion medium. The present study identified a potent antimicrobial activity of $bCGA_{344-358}$ in the low-micromolar range against bacteria, fungi and yeasts, without showing any haemolytic activity. Confocal laser microscopy demonstrated penetration of the rhodamine-nated peptide into the cell membranes of fungi and yeasts and its intracellular accumulation. Time-lapse videomicroscopy showed arrest of fungal growth upon penetration of the labelled peptide into a fungal filament. We identified several catestatin-containing fragments in the stimulated secretion medium of human polymorphonuclear neutrophils, suggesting the N-terminal sequence of catestatin ($bCGA_{344-358}$) (named cateslytin) as a novel component of innate immunity.

Key words. Chromogranin A; chromaffin secretory granule; catestatin; antimicrobial peptide; innate immunity; neuroimmunology.

The expanding number of patients at risk for invasive microbial infections intensifies the demand for antimicrobial agents. We have recently discovered new antimicrobial peptides generated from the processing of chromogranins, proenkephalin A and ubiquitin inside chromaffin granules of the adrenal medulla [1]. These peptides, co-secreted with catecholamines upon stimulation of the adrenal medulla [2–10], have highly conserved sequences. The antimicrobial activities of such peptides probably arose early in evolution. Because these peptides have a widespread distribution in endocrine, neuroendocrine, nervous cells and also immune cells, we suggest that they may play a role in inflammatory processes [6]. Chromogranins, a family of acidic soluble proteins, are widely distributed in endocrine cells, peripheral and central neurons [11]. Chromogranin A (CGA), the index member of this family, is routinely used as a diagnostic marker in clinical investigations [12, 13]. Because of the presence of eight to ten pairs of dibasic sites, CGA functions as a prohormone and generates bioactive peptides by posttranslational proteolytic processing [13, 14]. Catestatin, a small 21-amino-acid cationic peptide (net charge +5) within the bovine sequence ($bCGA_{344-364}$ RSMRLSFRARGYGFRGPGLQL) [15, 16], is a naturally occurring potent inhibitor (IC_{50} ~200–400 nM) of

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catecholamine release, acting at the nicotinic cholinergic receptor [17]. This peptide also displays potent vasodepressor activity and appears to diminish early in the course of the development of hypertension, even in the normotensive offspring of patients with the disease [18–20]. Recently, chromaffin cell secretion medium was demonstrated to contain not only 54-, 56- and 50-kDa catestatin-containing CGA intermediates including the intact catestatin ($bCGA_{344-364}$) [21], but also other short peptides derived from the catestatin domain of CGA ($bCGA_{343-368}$ and $bCGA_{332-361}$) that result from the combined actions of PC1 and PC2 (prohormone convertases) and the cysteine protease PTP (prohormone thiol protease) [21]. Catestatin has also been shown to act as a nicotinic-cholinergic antagonist in vivo inhibiting nicotine-evoked catecholamine secretion and gene transcription [22]. Recently, resequencing of the human CHGA gene from 180 individuals identified three naturally occurring human variants of catestatin: $Gly_{364}Ser$, $Pro_{370}Leu$, and $Arg_{374}Gln$ [23, 24]. These human variants displayed differential potencies towards inhibition of catecholamine release in vitro from PC12 cells [23, 24]. The rank order of potency for inhibition of catecholamine secretion was shown to be $Pro_{370}Leu$ > wild type > $Gly_{364}Ser$ > $Arg_{374}Gln$. The active core sequence of bovine catestatin, $CGA_{344-358}$, resides at the N terminus and displays highest nicotinic antagonism of catecholamine secretion [25]. In addition, this N-terminal active fragment triggered histamine release from rat pleural and peritoneal mast cells in vitro [26], analogous to the histamine-releasing effect of catestatin in vivo in rats [18]. The activation of histamine release by $bCGA_{344-358}$ suggested an interesting paracrine or endocrine role for this peptide in immunity. Because of the highly cationic nature of this peptide, a characteristic feature of the antibacterial compound, we hypothesized that catestatin and its N-terminal active domain ($bCGA_{344-358}$) may possess antibacterial activity. In the present in vitro study, we examined the antibacterial and antifungal activities of several peptides corresponding to bovine $bCGA_{344-358}$, wild-type human catestatin ($hCGA_{352-372}$) [27, 28], the two human variants ($Pro_{370}Leu$, $Gly_{364}Ser$) [23, 24] and several shorter peptides. We also tested the ability of these peptides to act against bacteria in the presence of drastic parameters (salts and elevated temperature) and to penetrate into mammalian cells, such as rabbit erythrocytes. Confocal laser microscopy demonstrated the interaction of rhodaminated $bCGA_{344-358}$ with fungi and yeast and determined the antimicrobial potency of these peptides. In addition, time-lapse videomicroscopy presented a detailed interaction of the labelled $bCGA_{344-358}$ with a filamentous fungus, *Neurospora crassa*. Previous findings demonstrate that human polymorphonuclear neutrophils not only store CGA but also secrete intact and processed forms upon stimulation [8]. In the present study, we show that a range of immunoreactive catestatin-containing peptides are released from human stimulated polymorphonuclear neutrophils (PMNs). The name of cateslytin has been assigned to this novel antimicrobial peptide corresponding to $bCGA_{344-358}$.

Materials and methods

Preparation of synthetic peptides

Bovine cateslytin ($bCGA_{344-358}$, RSMRLSFRARGYGFR), human catestatin ($hCGA_{352-372}$, SSMKLSFRARAYGFRGPGPQL) and the two variants $Gly_{364}Ser$ (SSMKLSFRARAYSFRGPGPQL) and $Pro_{370}Leu$ (SSMKLSFRARAYGFRGPGLQL) have been synthesized [29] in our laboratory and purified to >95% homogeneity by reverse phase high-performance liquid chromatography. For the fluorescence study, rhodaminated peptide was synthesized on an Applied Biosystems 432A peptide synthesizer using the stepwise solid-phase synthetic approach with FMOC chemistry, and the rhodamine was added as the first N-terminal residue of the polypeptidic chain. Peptide was automatically dissolved in diisopropylethylamine (Applied Biosystems) and treated by addition of 5,6-carboxytetramethylrhodamine (Fluka) and 1-hydroxybenzotriazole/o-benzotriazol-1-*y*l-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate (Applied Biosystems) as a coupling agent [9].

Preparation of anti-catestatin antibody

Polyclonal rabbit anti-catestatin antiserum recognizing the human catestatin region ($hCGA_{352-372}$: SSMKLSFRARAYGFRGPGPQL) was developed essentially as previously described [30, 31] with slight modification. This polyclonal antiserum recognizing the catestatin region of hCGA was purified on an Amersham Pharmacia Biotech HI Trap protein A column in 0.02 M sodium phosphate (pH 7.0), eluted with 0.1 M sodium citrate (pH 3.0), and the pH was then adjusted to 7.0 with Tris-HCl (pH 8.8).

Antibacterial and antifungal assays

The antibacterial and antifungal activities were tested as previously described [8]. Bacteria were precultured aerobically at 37°C in a Mueller-Hinton Broth medium, pH 7.3 (Difco Laboratories). The antibacterial activity was tested by measuring the inhibition of bacterial growth. Aqueous peptidic solutions (10 µl) were incubated in microtitre plates with 100 µl of a mid-logarithmic-phase culture of bacteria with a starting absorbance of 0.001 at 620 nm. Microbial growth was assessed by the increase of absorbance after 16 h incubation at 30°C. The A620 nm value of control cultures growing in the absence of peptide was taken as 100%. The following strains were tested: *Micrococcus luteus* (A270), *Bacillus megaterium* (MA), *Es-*

cherichia coli (D22), *N. crassa* (CBS 327OE54), *Aspergillus fumigatus, Nectria haematococca* (160.2.2), *Fusarium culmorum* (MUCL 30162), *F. oxysporum* (MUCL 909) and *Tricophyton mentagrophytes*. Yeast cells were precultured on a Sabouraud medium, and four strains were tested: *Candida albicans*, *C. tropicalis*, *C. glabrata* and *C. neoformans*. Filamentous fungi were grown on a five-cereal medium. Spores (final concentration 10^4 spores/ml) were suspended in a growth medium containing Potato Dextrose Broth (Difco, Becton Dickinson Microbiology Systems) at half strength, and yeast cells in Sabouraud medium (Biomérieux) with starting absorbance at 620 nm of 0.001. These media were supplemented with tetracycline (10 µg/ml) and cefotaxime (0.1 µg/ml). Fungal growth was assessed after an appropriate incubation period (24 or 48 h at 30 °C). Aliquots of peptide (10 µl) were incubated in microtitre plates with 90 µl of fungal spores and yeast cultures. Growth of fungi and yeast cells was evaluated by measuring the culture absorbance in a microplate reader at 595 and 620 nm, respectively. MIC is the minimal concentration inhibiting bacteria, filamentous fungi and yeast cell growth, and MIC_{100} is the minimal concentration completely inhibiting cell growth.

Confocal laser scan microscopy

The poly-L-lysine-coated chambered coverglass system (×8, Lab Tek, 0.8 cm^2; Nalge Nunc International), was covered with 180 µl of medium (one-half Potato Dextrose Broth or Sabouraud) containing *A. fumigatus* (104 spores/ml) or *C. albicans* (A620 nm = 0.001) at 30 °C for 21 or 16 h. Then, rhodamine-labelled $CGA_{344-358R}$ or the C-terminal fragment of chromogranin B ($CGB_{602-626R}$) was added to the culture medium at 30 °C. After incubation, chambers were washed with fresh culture medium, subsequently treated for 1 h with 4% paraformaldehyde in 0.12 mol/l sodium/potassium phosphate, pH 7.2, and covered with Elvanol-Mowiol. The fluorescence staining was monitored on a Zeiss confocal laser scanning microscope (LSM 510) equipped with a planapo oil (63×) immersion lens (numerical aperture, 1.4). Rhodamine emission was excited using the He/Ne laser 543-nm line, and the emission signal was filtered with a Zeiss long-pass 595-nm filter.

Time-lapse sequence videomicroscopy

To perform time-lapse videomicroscopy, fungi were embedded in a plasma clot [32] and grown at the bottom of the Petri dishes. Cultures were maintained at 30 °C in the chamber of the inverted microscope (Axiovert 200; Zeiss) equipped with a digital camera (Coolsnap *fx*; Roper Scientific). Images were taken every 30 min and movies were reconstructed using the Metaview software (Universal Imaging). The growth of fungus was recorded for 2–6 h to evaluate the normal growth of fungi in control conditions. The fluorescent peptides were directly added to the culture medium at a concentration of 0.1–1 µM and recording was performed during 2 to 6-h periods. To visualize the site of peptide incorporation, images were taken with with both a phase contrast objective and appropriate fluorescent filters.

Preparation and immunodetection of catestatin-containing fragments released from PMNs

Human PMNs were prepared to 98% homogeneity as previously described [33] from the buffy coat of healthy donors of either sex, kindly provided by the Centre de Transfusion Sanguine de Strasbourg (France). PMNs were suspended in a buffer solution containing 140 mM NaCl, 5 mM KCl, 1.1 mM $CaCl_2$, 0.1 mM EGTA and 10 mM Hepes, pH 7.3 at 5×10^6 cells/ml. Exocytosis of the content of the specific and primary granules was initiated at room temperature by application of 2.3 nM LukS-PV (leukotoxin class S Panton-Valentine) and 0.6 nM LukF-PV (leukotoxin class F Panton-Valentine), the two components of leukocidin produced from cultures of *Staphylococcus aureus* strain V8 (= ATCC 49775) harvested at the stationary phase [34]. The secretion was monitored by flow cytometry as previously described [35] and when complete, PMNs were centrifuged (800 g) for 10 min. Western blot analysis (12%, SDS-PAGE) was realized using anti-$hCGA_{352-372}$ antibody.

Results

The antimicrobial properties of $bCGA_{344-358}$ (cateslytin)

Catestatin ($bCGA_{344-364}$) contains an active core $bCGA_{344-358}$, which is an arginine-rich, cationic peptide (charge +5) [36]. We found that the fragment $bCGA_{344-358}$ inhibits the growth of the Gram-positive bacteria, *M. luteus* and *B. megaterium*, with a MIC of 0.8 µM and a MIC_{100} of 2 µM (table 1). Interestingly, this peptide is also active against Gram-negative bacteria, *E. coli D22* being killed with a MIC of 8 µM and a MIC_{100} of 15 and 50 µM at 37 and 41 °C, respectively (table 1). When tested against a filamentous fungus, $bCGA_{344-358}$ displays antifungal activity against *N. crassa* after incubation for 48 h at 30 °C, inhibiting the fungal growth with a MIC of 1.2 µM and MIC_{100} of 3.2 µM (table 1).

To complete this spectrum of activity, we tested the ability of $bCGA_{344-358}$ to affect the growth of other fungi and yeast cells (table 1). This bCGA-derived peptide was also highly active against a variety of filamentous fungi including *A. fumigatus, N. haematococca, F. culmorum, F. oxysporum, T. mentagrophytes* and several forms of *Candida*: *C. albicans, C. tropicalis, C. glabrata and C. neoformans* (table 1).

The haemolytic activity of this new antimicrobial peptide was tested on rabbit erythrocytes. At concentrations be-

Table 1. Antibacterial and antifungal activity of cateslytin ($bCGA_{344-358}$) [15, 16], the corresponding fragment in the human sequence ($hCGA_{352-372}$) [27, 28] and the two variants $P_{370}L$ and $G_{364}S$ [23, 24].

	$bCGA_{344-358}$		$hCGA_{352-372}$		$P_{370}L$		$G_{364}S$	
	MIC (µM)	MIC_{100} (µM)	MIC (µM)	MIC_{100} (µM)	MIC (µM)	MIC_{100} (µM)	MIC (µM)	MIC_{100} (µM)
Gram-positive bacteria								
M. luteus	0.8	2	5	15	2	10	1	2
B. megaterium	0.8	2	–	–	–	–	–	–
Gram-negative bacteria								
E. coli D22	8	15 50*	15	150	20	100	10	40
Filamentous fungi								
N. grassa	1.2	3.2	20	50	3	10	3	5
A. fumigatus	10	80	80	–	20	100	20	80
N. haematococca	0.2	0.8	–	–	–	–	–	–
F. culmorum	2	8	–	–	–	–	–	–
F. oxysporum	6	10	–	–	–	–	–	–
T. mentagrophytes	4	20	–	–	–	–	–	–
Yeast cells								
C. albicans	1.2	8	–	–	–	–	–	–
C. tropicalis	1.8	10	–	–	–	–	–	–
C. glabrata	8	30	–	–	–	–	–	–
C. neoformans	1.4	6	–	–	–	–	–	–

MIC, minimal concentration inhibiting bacteria, filamentous fungi and yeast cell growth; MIC_{100}, the minimal concentration completely inhibiting filamentous bacteria, fungi and yeast cell growth; * MIC_{100}, the minimal concentration completely inhibiting bacteria at 41 °C, –, not determined.

low 100 µM, no haemolytic activity could be observed. Accordingly, we have assigned the name cateslytin to this new non-haemolytic, antibacterial and antifungal peptide ($bCGA_{344-358}$, RSMRLSFRARGYGFR).

The antimicrobial properties of $hCGA_{352-372}$ and its two mutants $P_{370}L$ and $G_{364}S$

The sequence of catestatin $bCGA_{344-364}$ [15, 16] has been highly conserved during evolution, showing a high identity with human [27, 28], rat [37], mouse [38], pig [39], equine [40] and frog [41] forms (fig. 1). Recently, two residue replacements in the human catestatin sequence ($P_{370}L$ and $G_{364}S$) have been shown to produce different effects in their potency to inhibit nicotinic stimulation of catecholamine release from chromaffin cells [23]. The $P_{370}L$ variant increases catestatin activity 2.3-fold, whereas the $G_{364}S$ variant reduces catestatin activity 4.7-fold [23]. We examined the antibacterial and antifungal activities of $hCGA_{352-372}$ and its two variants. The synthetic peptide corresponding to the human sequence ($hCGA_{352-372}$) [28] displays antibacterial activity against *M. luteus* with a MIC of 5 µM (MIC_{100} of 15 µM) and against *E. coli* with a MIC of 15 µM (MIC_{100} of 150 µM) (table 1). The two human variants $P_{370}L$ and $G_{364}S$ display potent antibacterial activity against *M. luteus* with a MIC of 2 and 1 µM, respectively, and against *E. coli* with a MIC of 20 and 10 µM, respectively (table 1). However, the most potent active peptide corresponds to the bovine sequence.

Analysis of the antimicrobial properties of cateslytin and several derived peptides

Cateslytin corresponds to a cationic sequence with a global net charge of +5 with five basic residues R_{344}, R_{347}, R_{351}, R_{353} and R_{358} and five hydrophobic residues M_{346}, L_{348}, F_{360}, Y_{355} and F_{357}. We investigated its antibacterial activity against *M. luteus* in the presence of NaCl (0–150 mM) in addition to the regular medium and showed that in these conditions, cateslytin is able to completely kill bacteria at concentrations lower than 10 µM (table 2). To demonstrate that the antimicrobial activity of cateslytin was not due to non-peptidic material, this peptide was submitted to tryptic digestion for 18 h at 37 °C (trypsin/peptide ratio 1/100 in 100 mM sodium bicarbonate buffer pH 7.8). Using matrix-assisted laser desorption ionization time-of-flight analysis, we identified two predominant peptides ARGYGFR ($CGA_{352-358}$; MW 827 Da) and GYGFR ($CGA_{354-358}$; MW 601 Da). The tryptic digest

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bCGA344-364   [15,16] R S M R L S F R A R G Y G F R G P G L Q L
hCGA352-372   [27,28] S S M K L S F R A R A Y G F R G P G P Q L
P370L         [23,24] S S M K L S F R A R A Y G F R G P G L Q L
G364S         [23,24] S S M K L S F R A R A Y S F R G P G P Q L

rCGA367-387   [37]    R S M K L S F R A R A Y G F R D P G P Q L
mCGA364-384   [38]    R S M K L S F R T R A Y G F R D P G P Q L
pCGA343-363   [39]    R S M K L S F R A P A Y G F R G P G L Q L
eCGA361-375   [40]    R S M K L S F R A R A Y G F R G P G L Q L

fCGA318-332   [41]    R S M K I P T K D Q K Y - - - E P A S E E
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Figure 1. Sequence alignment of bovine $CGA_{344-358}$ with corresponding sequences of catestatin fragments from several species. Human catestatin, ($hCGA_{352-372}$) and the two human variants $P_{370}L$ and $G_{364}S$, rat ($rCBA_{367-387}$), mouse ($mCGA_{364-384}$), pig ($pCGA_{343-363}$), equine ($eCGA_{361-381}$) and frog (fCGA $_{318-335}$).

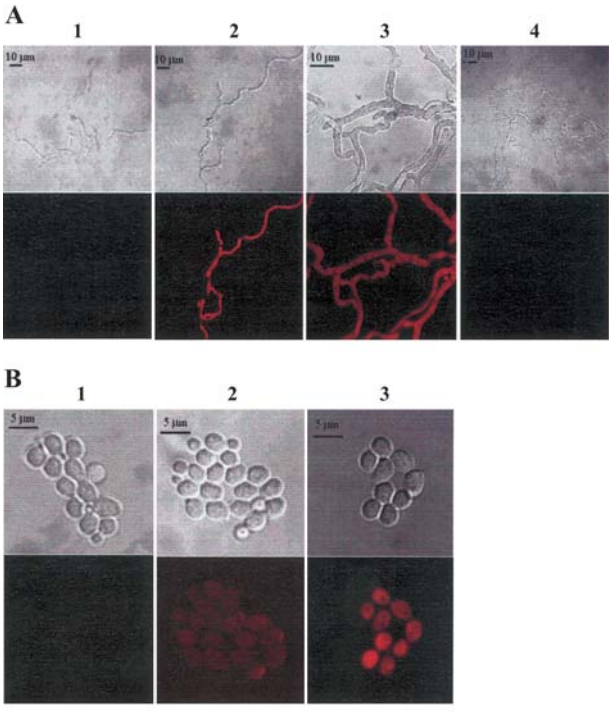

Figure 2. Phase-contrast and fluorescence confocal laser micrographs of *A. fumigatus* and *C. albicans*. (*A*) After 21 h in culture medium (30 °C), *A. fumigatus* was examined: 1, in the absence of synthetic peptide; 2, after incubation for 2 min with 1 µM of rhodamine-labelled $bCGA_{344-358}$; 3, after incubation with 5 µM peptide for 1 h; 4, after incubation, as control, with 10 µM of inactive labelled $CGB_{602-626R}$ for 1 h. (*B*) *C. albicans* was examined after incubation for 16 h in cultured medium (30 °C): 1, in the absence of rhodamine-labelled synthetic peptide; 2, after incubation with 1 µM peptide for 2 min; 3, after incubation with 5 µM peptide for 1 h.

was completely inactive against *M. luteus*, indicating that the two short peptides $bCGA_{352/354-358}$ were inactive at a concentration lower than 200 µM (table 2). To identify the structural features important for the antibacterial activity of cateslytin, we prepared two synthetic cationic peptides $CGA_{348-358}$ and $CGA_{344-351}$ (table 2) corresponding to its N- and C-terminal domains. The two peptides with four and three hydrophobic residues, respectively, and a global positive charge of +3, displayed an antibacterial activity against *M. luteus* with a MIC_{100} of 20 µM.

Confocal laser microscopy analysis of the interaction of cateslytin with *A. fumigatus* and *C. albicans*

In order to understand the molecular mechanism involved in the antifungal activity of cateslytin, we used confocal microscopy to analyse the interaction of the synthetic rhodamine-labelled cateslytin ($CGA_{344-358R}$) with fungal and yeast membranes of *A. fumigatus* and *C. albicans*. We verified that the rhodamine-labelled $CGA_{344-358R}$ possesses comparable antifungal activity to that of the unlabelled peptide. *A. fumigatus* fungal spores were first incubated at 30 °C for 21 h in the absence of peptide (fig. 2A1). Rhodaminated cateslytin at 1 µM

Table 2. Sequences and antibacterial properties of cateslytin and several derived peptides.

Location	Sequence	h	Positive charge number	MIC_{100} (µM)	NaCl (µm)
$bCGA_{344-358}$	RSMRLSFRARGYGFR	5	5	2	0
	+ h +h h+ + h h+			5	25
				5	50
				10	150
$bCGA_{352-358}$	ARGYGFR	2	2	–	
$bCGA_{354-358}$	GYGFR	2	1		
$bCGA_{348-358}$	LSFRARGYGFR	4	3	20	
$bCGA_{344-351}$	RSMRLSFR	3	3	20	

Sequence of cateslytin ($bCGA_{344-358}$), the two tryptic fragments $bCGA_{352-358}$, $bCGA_{354-358}$ and the two synthetic peptides $bCGA_{348-358}$ and $bCGA_{344-351}$; h and + indicate hydrophobic and basic residues, respectively. The antibacterial activity of the different peptides was evaluated by their ability to completely inhibit the growth of *M. luteus* (MIC_{100}). The activity of cateslytin was evaluated in the presence of NaCl (25, 50 and 150 mM). The tryptic digest containing the peptides $bCGA_{352-358}$, $bCGA_{354-358}$ was unable to display antibacterial activity (–), at a concentration lower than 200 µM. The antibacterial activity of the two synthetic derived peptides $bCGA_{348-358}$ and $bCGA_{344-351}$ is also indicated.

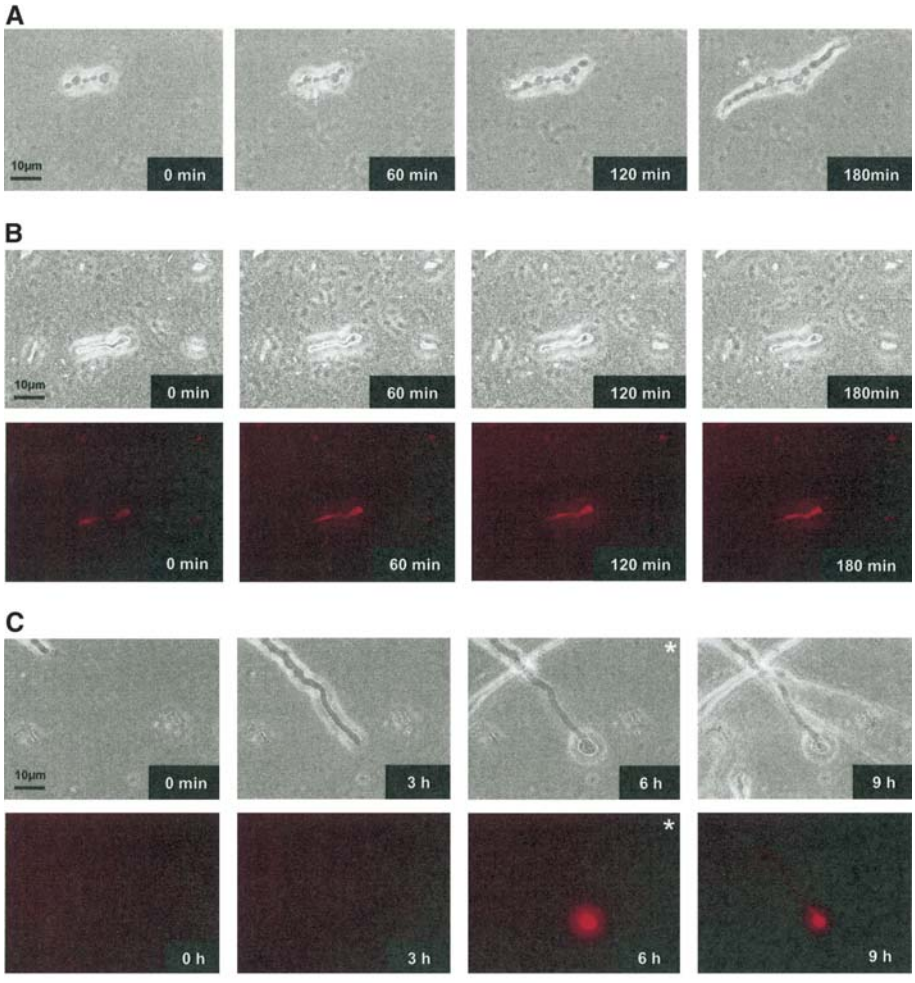

Figure 3. Time-lapse videomicroscopy analysis of *N. crassa* growth in the presence of a low concentration of rhodaminated bovine $CGA_{344-358}$. (*A*) Morphological analysis. The time elapsed between two frames is 1 h, with the last frame corresponding to the morphological stage after 3 h. (*B*) Penetration of rhodaminated bovine $CGA_{344-358}$ (1 µM) into a short fungus after 30 min of incubation. The peptide enters at the two ends and completely stops fungal growth. (*C*) Penetration of rhodaminated bovine $CGA_{344-358}$ (1 µM) into a hyphal extension after 30 min incubation. The time elapsed between two frames is 3 h. When the peptide penetrates into the fungus as indicated with the asterisk (6 h), it completely blocks growth and induces the formation of a vesicular structure at the tip.

was visible in the inner compartment after 2 min of incubation, indicating that cateslytin is able to rapidly and efficiently penetrate the cell wall (fig. 2A2). When the concentration of rhodaminated cateslytin was increased (5 µM) and the incubation time was prolonged (1 h), unlabelled vacuoles became visible without fluorescence on the septum separating two cells (fig. 2A3). Thus, cateslytin accumulates within fungi, inducing formation of unlabelled vacuoles. The fluorescent inactive $CGB_{602-626R}$ peptide was used as a control (fig. 2A4) [9]. We also examined the spores of *C. albicans* (fig. 2B), prepared by a first incubation for 16 h without peptide followed by a second incubation for 1 h at 30 °C with rhodaminated cateslytin. Compared with control experiments in the presence of fluorescent inactive $CGB_{602-626R}$ peptide (fig. 2B1), the fluorescent cateslytin was detectable in cytoplasm from a low concentration (1 µM) within 2 min (fig. 2B2). Increasing the concentration of peptide (5 µM) and incubation time (1 h), the amount of cateslytin that accumulated into the cytoplasm was enhanced without provoking cell lysis (fig. 2B3). This suggests that $CGA_{344-358}$ can cross the yeast cell wall and accumulate in the inner part of the cell.

Time-lapse videomicroscopy of fungal growth in the presence of a low concentration of rhodaminated cateslytin

To examine further the antimicrobial effect of cateslytin, time-lapse videomicroscopy of developing fungi was performed in live conditions to follow the growth of hyphal tips of *N. crassa* with or without rhodaminated cateslytin in the culture medium (fig. 3). In absence of peptide, *N. crassa* grows normally during the periods of recording, as shown by the extension of the fungi and the

formation of new filaments (fig. 3A). In contrast, the growth and development of nascent fungus that have accumulated rhodaminated cateslytin were blocked, with no further extension (fig. 3B). We observed that the growth was arrested as soon as the peptide penetrated into the developing fungus (fig. 3C). After diffusion of the peptide in the culture medium and through the plasma clot, the penetration occurred along the fungus from the hyphal tip and produced the induction of terminal varicosity (fig. 3C).

Presence of catestatin-derived peptides in the secretions from PMNs

The secretions released from human PMNs were submitted to immunodetection in Western blot (fig. 4A) with a specific anti-catestatin antibody [30, 31]. We detected several immunoreactive N-terminal CGA-derived fragments. A predominant band located at 60 kDa corresponds to the fragment CGA_{79-439} generated after the removal of the vasostatin-I fragment (CGA_{1-76}), and the band located at 70 kDa corresponds to the intact protein. The other bands immunodetected at 40, 27, 17 and 15 kDa may correspond to fragments previously reported to be involved in proteolytic degradation of CGA excreted in urine of patients with carcinoid tumors [42]. The shortest immunodetected catestatin-containing fragment corresponds to the sequence $hCGA_{340-394}$. As indicated in figure 4B, this fragment may be processed according to previous studies on the homologous bovine sequence [14, 17, 21].

Discussion

Antimicrobial peptides are classified by their mode of action, with their primary target being the microbial membrane [43]. While one group of peptides may lyse the organism via disruption of the membrane and/or formation of aqueous pores, the other group may interfere with the biosynthesis of cellular components such as glucan or chitin, essential to cell wall synthesis [44]. Despite extensive research, the mode of action of antimicrobial peptides has remained elusive [45–48]. The confocal microscopy analysis of the interaction of cateslytin ($bCGA_{344-358}$) with *A. fumigatus* and *C. albicans* demonstrates that cateslytin (in the low micromolar range) readily crosses the cell membrane and rapidly reaches the intracellular space (fig. 2). Consistent with the above findings, the time-lapse experiments with *N. crassa* documented a highly dynamic mode of penetration of the rhodaminated cateslytin into the live fungal cells (fig. 3). Penetration of the rhodaminated peptides into the tip of the growing fungus readily inhibits fungal growth. Of note, peptide penetration in small fungi (three cells and expressing a slow growth rate) takes place at both ends of the fungus, ultimately arresting growth. In contrast, in larger fungi with a higher growth rate, the peptide concentrates at one end before progressive diffusion. In all cases, the penetration of the peptide at the distal tip induces the formation of vesicles and conidia that correlates with the inhibition of fungal growth. Because of its

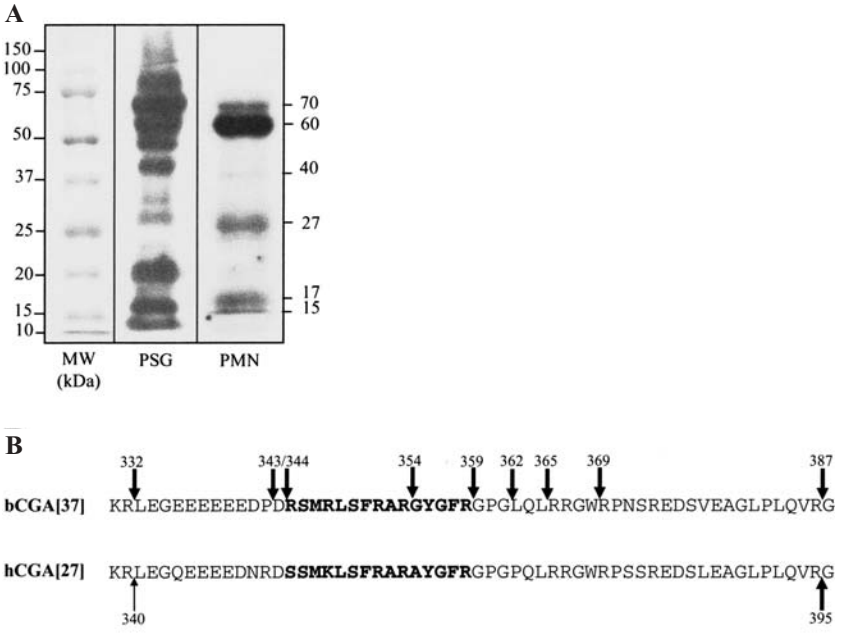

Figure 4. Identification of immunoreactive catestatin components released from stimulated PMNs. (*A*) Western blot analysis (12%, SDS-PAGE) with anti-$hCGA_{352-372}$ antibody. 1st lane, molecular-mass standards; 2nd lane, soluble proteins from chromaffin granular matrix; 3rd lane, secretions from human PMNs. Electrophoresis and Western blot were performed as described in Materials and methods. (*B*) Natural processing of bovine catestatin domain $bCGA_{330-387}$ and the human corresponding sequence $hCGA_{338-395}$. Arrows with number represent natural proteolytic cleavage sites previously reported.

extremely fast rate of tip growth (>1 µm/s) [43], *N. crassa* represents a potent system to study cell polarization. Of interest is to note that the antimicrobial activity of bovine cateslytin is more potent than that of the three corresponding human sequences, thereby establishing the N-terminal arginine residue as being crucial for the antimicrobial activity. In addition, we have shown that the N-terminal sequence $bCGA_{344-351}$ is crucial for the antibacterial activity (table 2).

We conclude that the natural cateslytin with a cationic arginine-rich amphiphilic peptide displays potent antimicrobial activity against bacteria, fungi and yeast cells. This peptide rapidly passes through the cell membrane, accumulates in the inner part of the cells and possibly acts on intracellular targets. Because of the low toxicity (at micromolar levels) of cateslytin against mammalian cells, we suggest that this novel antimicrobial peptide represents a relevant component of innate immunity and may serve as a crucial reagent for the development of potential drugs or design models for new agents for antibiotic-resistant micro-organisms.

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