Evolution of the Primate Cathelicidin

CORRELATION BETWEEN STRUCTURAL VARIATIONS AND ANTIMICROBIAL ACTIVITY*§

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Igor Zelezetsky*,1,2, Alessandra Pontillo5§, Luca Puzzi§, Nikolinka Antcheva§, Ludovica Segat§, Sabrina Pacor§, Sergio Crovella§, and Alessandro Tossi‡3

From the Departments of *Biochemistry, Biophysics and Macromolecular Chemistry, §Reproduction and Development Sciences, and ¶Biomedical Sciences, University of Trieste, Trieste, I-34127 Italy

Cathelicidin genes homologous to the human CAMP gene, coding for the host defense peptide LL-37, have been sequenced and analyzed in 20 primate species, including Great Apes, hylobatidae, cercopithecidae, callithricidae, and cebidae. The region corresponding to the putative mature antimicrobial peptide is subject to a strong selective pressure for variation, with evidence for positive selection throughout the phylogenetic tree relating the peptides, which favors alterations in the charge while little affecting overall hydrophobicity or amphipathicity. Selected peptides were chemically synthesized and characterized, and two distinct types of behavior were observed. Macaque and leaf-eating monkey RL-37 peptides, like other helical antimicrobial peptides found in insect, frog, and mammalian species, were unstructured in bulk solution and had a potent, salt and medium independent antimicrobial activity in vitro, which may be the principal function also in vivo. Human LL-37 and the orangutan, hylobates, and callithrix homologues instead showed a salt-dependent structuring and likely aggregation in bulk solution that affected antimicrobial activity and its medium dependence. The two types of peptides differ also in their interaction with host cells. The evolution of these peptides has thus resulted in distinct mechanisms of action that affect the direct antimicrobial activity and may also modulate accessory antimicrobial functions due to interactions with host cells.

Host defense peptides (HDPs), produced by epithelial and phagocytic cells, are an important component of innate immune defense at mucosal surfaces. Among these are the cathelicidin family of HDPs, which have been identified in several mammalian and non-mammalian species, and that are characterized by conserved pro-regions carrying highly variable antimicrobial sequences (1, 2). Besides the direct capacity to inactivate microbes, some of these cathelicidin antimicrobial peptides have also been found to act as immune modulators and mediators of inflammation, influencing diverse processes such as cell proliferation and migration, wound healing, angiogenesis, and the release of cytokines and histamine (3). For these reasons, they qualify as prototypes of innovative multifunctional drugs that may be used to treat infection and/or modulate immune response (4).

Cathelicidins all include N-terminal signal peptides followed by cathelin-like domains (the pro-region), which are conserved across species, followed by the highly diverse C-terminal antimicrobial domains. In mammals, their structures range from cysteine-bridged hairpins to linear tryptophan-rich, proline-rich, or α-helical molecules, and their sizes range from a dozen to over 80 residues (1, 2, 5). The helical group is the most widespread, and its members generally display a broad-spectrum bactericidal activity (6, 7). Circular dichroism studies indicate that these peptides are generally unstructured in bulk solution, and adopt a helical conformation only in contact with membrane-like environments, whereas most of the published evidence indicates that they act by disrupting the bacterial membrane (7–9). This type of cathelicidin HDP has been identified in rodents (rabbit rCAP-18, mouse and rat CRAMP, and guinea pig CAP-11), primates (human LL-37 and rhesus RL-37), bovids (bovine BMAP-27, -28, and -34, sheep SMAP-29 and -34, and goat MAP-28 and -34), horse (eCATHI-1 to -3), and pig (PMAP-23, -36, and -37), and a putative member has recently been identified also in dog (2).

The only cathelicidin gene present in humans (CAMP) is expressed in inflammatory and epithelial cells, and its product (hCAP-18) contains a 30-residue signal region, a 103-residue polypeptide corresponding to the conserved cathelin-like pro-region, and the 37-residue peptide LL-37 at the C terminus, which becomes antimicrobially active on release from the pro-region by the action of proteinase 3 (10). The mature HDP, which has been identified in plasma, in the airway surface fluid and in wound secretions, is induced by inflammatory or infec-

methyluronium hexafluorophosphate; TBTU, (b-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; PyBOP, 1H-benzotriazol-1-yl-oxy-tris(pyrrrolidino)phosphonium hexafluorophosphate; TFE, trifluoroethanol; NWM, New World Monkey.
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tious stimuli and displays membrane-directed antimicrobial activity, in vitro (1, 3). However, this activity seems to be quite medium sensitive, and accompanied by a relatively high cytotoxicity, possibly related to its tendency to aggregate to form multimeric helical bundles (11, 12). Furthermore, in line with the widening role of antimicrobial peptides as effector molecules of innate immunity, LL-37 was also found to bind to formyl peptide receptor-like 1 (FPR1), a G protein-coupled, 7-transmembrane cell receptor found on macrophages, neutrophils, and subsets of lymphocytes (13). Thus, apart from being an endogenous cytolytic antibiotic providing a first line of host defense, it also acts as a multifunctional immune mediator by modifying the local inflammatory response, and helping to activate adaptive immunity. In this respect, LL-37/hCAP-18 has also been found to induce a functionally important direct effect on endothelial cells, so that it links inflammation and host defense with angiogenesis (14). The cathelin-like N-terminal pro-region, after release of the HD/P by proteolytic cleavage, can also apparently contribute to innate host defense through inhibition of bacterial growth and limitation of cysteine-protease-mediated tissue damage (15). As these dual functions are complementary to those of LL-37, the human cathelicidin represents an elegant multifunctional effector molecule for innate immune defense of the skin.

The human CAMP gene consists of four exons (Fig. 1) and is localized on chromosome 3p21.3 (16). It contains several potential binding sites for transcription factors (acute-phase response factor and nuclear factor for interleukin-6 expression) that are possibly involved in the regulation of gene expression (16, 17). To study the evolution of this gene, we have sequenced the homologues of the entire CAMP gene coding region in 20 non-human primate species and compared it with the human sequence. This has provided information on how variations in the sequence of the C-terminal HD/P affect its structural characteristics, and how this correlates with its functional characteristics, and in particular with the capacity to directly inactivate different types of bacteria and yeast. Moreover, we have investigated whether any of these variations could be due to a positive selective pressure, possibly derived from exposure to different biotas and/or pathogens.

EXPERIMENTAL PROCEDURES

Materials—2-Chlorotrityl chloride resin, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTO), and Fmoc (9-(9-fluorenylethoxycarbonyl)-protected amino acids were from Applied Bio- tech Italy (Milan, Italy). HBTBenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) was from Novabiochem (Darmstadt, Germany). Mueller-Hinton broth and Bacto-agar were obtained from Difco Laboratories (Detroit, MI); o-nitrophenyl-β-D-galactopyranoside, lysozyme, lysostaphin, and bovine serum albumin were from Sigma, and bovine serum albumin was from Calbiochem (Darmstadt, Germany). All other reagents were of analytical grade. Buffers were prepared in double glass-distilled water.

Non-human Primate DNA Samples—We have collected biological samples (muscles from deceased animals or hairs) for different non-human primate species at the Negara Zoo, Malaysia (Kuala Lumpur), and at the Mulhouse Zoo, France. Other DNA samples were obtained at the genomic primates DNA bank of the genetic service of the Department of Reproduction and Development Sciences (University of Trieste). A total of 20 non-human primate species and subspecies, including several individuals per species were analyzed (numbers indicated in parentheses). These were, among the catarrhini: three great Ape species, Pan troglodytes, chimpanzee (3), Gorilla gorilla (3); and Pongo pygmaeus, orangutan (4); five hylotbatidae (gibbon) species: Hylolobates concolor (5), H. concolor gabriellae (2), Hylolobates lar (2), H. concolor leuconyenus (2), H. moloch (3); seven cercopithecidae (Old World Monkey) species, including five cercopithecinae species: the macaques M. fascicularis (25), M. mulatta (rhesus macaque) (7), M. tonkeana (5), the baboon Papio anubis (3), and the vervet monkey Cercopithecus aethiops (15); and two colobine species (leaf-monkeys): Presbytis cristata (5) and P. obscurus (3). Among the platyrrhini (New World Monkeys), there were two callithricidae species, the marmoset Callithrix jacchus (30) and the tamarin Saguinus oedipus (2); three cebidae, including two spider monkey subspecies: Ateles fusciceps (3) and A. fusciceps robustus (1) and the capuchin monkey Cebus capucinus (5).

DNA Extraction, PCR Amplification, and Sequencing—Genomic DNAs were extracted from the muscle tissue using the GenomePrep DNA extraction kit (Amersham Biosciences). DNA was extracted from hairs using the Chelex 100 method following standard protocols (18). Genomic amplification of LL-37 was performed using primers designed on the basis of the published human sequence (GenBank accession number NT_022517). Primer sequences were as follows (see Fig. 1): 1F, first exon, forward primer: 5′-AAGCAATAGCCAGGGCTAAAGCAAACCC-3′; 1R, first exon, reverse primer: 5′-GGCATCT- CCCCCTTTAAATGTGGTCC-3′; 2F, second exon, forward primer: 5′-GGGTAAGAATGGCTAGCTT-3′; 2R, second exon, reverse primer: 5′-TGTTGACGCGGTATGTTG-3′; 3F, third exon, forward primer: 5′-GAGGTCTATGGCATAATGTTCT-3′; 3R, third exon, reverse primer: 5′-AGAGTTGGACCATCCAAATGTTGCTAAT-3′; 4F, fourth exon, forward primer: 5′-TCATATCTCCGGGAATGTCTGTTT-3′; 4R, fourth exon, reverse primer: 5′-CTTCTCTCCTTCACCCACACTG-3′.

FIGURE 1. Schematic representation of the hCAMP gene. White boxes, 5′- and 3′-untranslated region (UTR); hatched box, signal sequence; gray box, pro-region; black box, mature peptide. Scissors indicate sequentially events of enzymatic cleavage that finally produce the mature HD/P. LL-37. Labeled arrows indicate primers used for amplification of prime CAMP exons and sequencing (see “Materials”).
The PCRs were carried out in a GeneAmp PCR System 9700 (Applera Genomics, Foster City, CA) with 1 unit of Taq Gold (Applera Genomics), 0.4 mM dNTPs and variable concentrations of MgCl₂ (from 1.5 to 2.5 mM). The amplification conditions were 30 s at 95 °C, 30 s at 54 °C, and 30 s at 72 °C for 45 cycles. PCR products were observed, under UV light, in a 2% agarose gel, stained with ethidium bromide. DNA sequencing of the amplicons was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 2.0 (Applied Biosystems). DNA sequences were detected and analyzed on an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequence Analyses and Comparisons—Multiple alignments of the nucleotide and amino acid sequences were performed using the software CLUSTAL X. A phylogenetic tree was then obtained by Neighbor Joining analysis, using the PHYLIP software package, with the mouse CRAMP sequence as the outgroup. Maximum likelihood (ML) analysis was performed with the PAML software package (19), comparing the log-likelihood ratios of the data using different NSites models (20). This type of analysis is able to identify amino acid residues with high posterior probabilities (greater than 0.95) of having evolved under positive selection. ω ratios for the tree were calculated by different models (free ratio, two and three ratio) (21).

The global hydrophobicity and the amphipathicity for each deduced putative HDP sequence were calculated using a hydrophobicity index (H) scale derived from the normalized and filtered consensus of 163 published scales (22) arbitrarily ranged between maximum values of +10 for Phe and −10 for Arg. The hydrophobicity is given as the mean per residue value \( H = \sum H_i / 37 \). The mean hydrophobic moment \( \mu H_{max} \) was calculated as described by Eisenberg et al. (23). The relative amphipathicity \( \mu H / \mu H_{max} \) for each peptide was then determined with respect to the value of the maximum hydrophobic moment for a perfectly amphipathic, 18-residue peptide composed only of Phe and Arg \( \mu H_{max} = 6.4 \) with our scale. This relative measure of amphipathicity is less likely to vary according to the scale used than an absolute value.

Peptide Synthesis and Characterization—Solid phase peptide syntheses were performed on a thermostated PE Biosystems Pioneer® automated peptide synthesizer loaded with 2-chlorotrityl chloride resin (substitution 0.22–0.27 milliequivalent/g). Insertion of the C-terminal amino acid was carried out manually according to the protocol provided in the synthesis notes section of the 2003 Novabiochem catalog (section 2.17). A 4-fold excess of 1:1:1.7 Fmoc-amino acid/TBTU/diisopropylethylamine and a version of the anti-aggregation solvent “magic mixture” (dimethylformamide/N-methylpyrrolidone (3:1, v/v), 1% Triton X-100, 1 mM ethylene carbonate) were used for each coupling step. Double coupling with HATU or PyBOP was carried out at all positions. Peptides were cleaved from the resin and deprotected using a mixture consisting of trifluoroacetic acid, water, and trisopropylsilane (95:2.5:2.5, by volume).

The crude peptides were purified with a Waters Delta-Pak® C₁₈ column (5 μm, 300 Å, 25 mm × 100 mm) and the fraction containing the correct peptide was purified by ESI-MS (PE Sciex API 1). Peptide concentrations were estimated gravimetrically and confirmed using both spectrophotometric determination of phenylalanine \( \epsilon_{277} = 195.1 \) and colorimetric determinations using the BCA protein assay kit (Pierce) and the ninhydrin assay.

Circular Dichroism—Structuring was probed by CD spectroscopy on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan), using 2-mm path length quartz cells and a default peptide concentration of 40 μM, under a series of conditions. All spectra are the mean of at least two trials, each with the accumulation of three scans. Helical content was estimated from the ellipticity at 222 nm, according to Chen et al. (24).

Antimicrobial Activity Assays—The antimicrobial activity of the synthetic peptides was determined against Escherichia coli ML-35, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus 710A, Enterococcus faecalis A16, and a Candida albicans clinical isolate as the minimum inhibitory concentration using a microdilution susceptibility test as described previously (25). Briefly, serial 1:1 dilutions of each peptide were made in 96-well microtiter plates, to a final volume of 100 μl of tryptic soy broth (TSB) (5% or 20% (v/v) in SPB, pH 7.4, or in PIL buffer, pH 7.3) with 10⁵ colony forming units/ml of bacteria in logarithmic phase. Plates were incubated at 37 °C overnight and the minimum inhibitory concentration was considered as the first well without visible growth. For antifungal assays, fungi were cultivated on solid Sabouraud/dextrose agar medium, and minimum inhibitory concentration values were determined in Sabouraud/dextrose medium (5 or 20% (v/v) in SPB or PIL buffers). The kinetics of bacterial killing was confirmed against both E. coli ML-35 and S. aureus 710A in the logarithmic phase (10⁴ colony forming units/ml in PBS incubated at 37 °C). At different times, 50 μl of the bacteria/peptide suspension was diluted several fold in ice-cold PBS, and the solution plated on nutrient agar and incubated overnight to allow colony counts.

The permeabilization of the outer membrane of E. coli by the peptides was evaluated by following the unmasking of the periplasmic hydrolytic enzyme β-lactamase, using extracellular CENTA as substrate, whereas that of the cytoplasmic membrane by unmasking cytoplasmic β-galactosidase activity using extracellular o-nitrophenyl-β-D-galactopyranoside as substrate, as described previously (26). The β-galactosidase constitutive, lactose-permease-deficient E. coli ML-35 pYC strain was used (10⁷ colony forming units/ml), exposed to a 5 μM peptide concentration and 1.5 mM of either substrate, in PBS with or without addition of 20% (v/v) TSB.

Permeabilization of the cytoplasmic membrane of S. aureus 710A was instead evaluated by following unmasking of the cytoplasmic 6-phospho-β-galactosidase activity as described previously (27), using freshly synthesized o-nitrophenyl-β-D-galactopyranoside-6P and 5 μM peptide concentrations. Bacteria treated with lysostaphin (30 μg/ml) and egg white lysozyme (30 μg/ml) were taken to be 100% permeabilized.

Cytotoxicity Assays—Hemolytic activity was determined as lysis of erythrocyte membranes by monitoring the release of hemoglobin at 415 nm, from 0.5% human erythrocyte suspensions (0.2 ml) in relation to complete (100%) hemolysis as determined by addition of 0.2% Triton X-100. Flow cytometric analysis was performed with an XL instrument (Coulter Instruments), suspensions of 10⁶ lymphocytes in 1 ml of PIL buffer were exposed to peptides for 60 min at 37 °C as described previously (28). Fluorescence data from damaged cells showing
permeability to PI was acquired in a monoparametric histogram (emission wavelength /H11005 612 nm) and submitted to the Student-Newmann-Keuls test of variance.

RESULTS

Amino Acid Sequence Analyses—The amino acid sequences for primate CAP-18 were deduced from the determined sequences of primate genes orthologous to CAMP (see supplemental Fig. S1). Like hCAP-18, the open reading frame in all cases consists of a putative 30-residue N-terminal signal sequence, the 103-residue pro-region (cathelin-like domain), with the four characteristic conserved cysteine residues involved in two disulfide bridges, and the mature C-terminal HDP of 37 amino acids (see Fig. 2 for the aligned, 170-residue full sequences). Of these, only the human (16) and rhesus macaque (29, 30) sequences had been reported previously. Our rhesus sequence is in agreement with that of Zhao et al. (29).

Among the deduced sequences, those from the Great Apes and the hylobatidae showed the highest similarity with the human one (91–96 and 90–95%, respectively), followed by the Old World Monkeys ( cercopithecidae, 91–92%) and then the New World Monkeys (78–84%). As expected, the majority of amino acid substitutions involved the putative antimicrobial HDP region, and only the peptide from our closest relative, the chimpanzee (PTR), is identical to human. Variation in the 37-residue HDP region with respect to other species ranges from 5 to 8% for the other Great Apes (gorilla GGO and orangutan PPY), 16–24% for the hylobatidae (gibbons HCO, HCG, HCL, HMD, and HLE), 30–32% for cercopithecidae (the macaques MMU, MFA, and MTO, the baboon PPA, the vervet CCA, and the silvered and dusky leaf monkeys PCR and POB), and over 43% for platyrrhine monkeys of the callithricidae (CJA and SOE) and cebidae families (CCA, AFU, and AFR). These substitutions, however, tend to be conservative, in the sense that the overall polar/hydrophobic residue distribution is maintained, as can best be appreciated by mapping them on a helical wheel (Edmundson) projection of the human peptide (Fig. 3).

All the primate cathelicidin HDPs show an amphipathic distribution of residues over the whole sequence, with well defined polar and hydrophobic sectors. Residue variations between them tend to maintain this arrangement, as evidenced also by a limited variation in the relative amphipathicity (Fig. 2). The charge instead varies remarkably, from +4 in the orangutan and Ateles species to +10/+11 in two cercopithecidae species, the Asian leaf monkey Presbytis obscurus, and the African vervet monkey Cercopithecus aethiops, respectively.

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**FIGURE 2. Alignment of primate cathelicidin-deduced amino acid sequences.** Dots indicate amino acid identity to the human sequence. The gray bars highlight the conserved cysteine residues. The charge (q), mean residue hydrophobicity (H), and relative amphipathicity (Hrel) of the putative HDP are provided (histidine is considered as neutral). A consensus sequence is shown below the HDP sequences, with completely conserved residues shaded in black, highly conserved residues shaded in gray, and most frequent residues shown in lowercase. + indicates a basic residue (Arg or Lys) and x a non-conserved position. Species are: Homo sapiens (HSS); P. troglodytes (PTR); G. gorilla (GGO); P. pygmaeus (PPY); H. lar (HCL); H. moloch (HMD); H. leucogenys (HLE); H. concolor (HCO); H. concolor gabriellae (HCG); M. mulatta (MMU); M. fascicularis (MFA); M. tonkeana (MTO); P. anubis (PPA); C. aethiops (CAE); P. cristata (PCR); P. obscurus (POB); A. fusciceps (AFU); A. fusciceps robustus (AFR); C. capucinus (CCA); S. oedipus (SOE); C. jaccus (CJA).
possible due to the interchanging of cationic, anionic, and neutral polar residues at specific positions (Fig. 3). It can thus be hypothesized that evolution works to vary the charge of these peptides while conserving the amphipathic structure.

From positional residue frequencies in the sequence alignment, it is possible to derive a consensus sequence (Fig. 2, bottom) in which about half the residues are quite conserved, and interspersed throughout the sequence cluster more at the C terminus. A comparison of selected primate peptides with the HDPs from other mammalian cathelicidins that may be evolutionarily related to LL-37 (2) are shown in Fig. 4. Conserved residues tend to coincide. A central cluster, corresponding to residues 19–26 and bordering residues, is particularly conserved in all represented species. Other residues in stretches 9–13 and 30–36 also show some conservation with respect to rodents and dog sequences. It could thus be further hypothesized that these peptides may all have arisen from a common ancestral molecule, in which certain stretches have been conserved during evolution, whereas others have been allowed to vary. In this respect, the C-terminal part of the primate peptides, which is most conserved and coincides with conserved stretches in other mammals, may have a central structural and/or functional role.

**HDP Synthesis and Characterization**—Six primate HDP sequences were selected for synthesis and characterization (Table 1). These were, apart from the reference human hssLL-37, the orangutan ppyLL-37, as the most different Great Ape peptide, and also one with the lowest charge; the gibbon hmdSL-37, as representative of the hylobatidae, the rhesus macaque mmuRL-37 and the highly cationic pobRL-37 as representatives of the cercopithecidae, and cjaRL-37 as representative of the New World Monkeys.

The capacity of the peptides to structure was then determined in a number of different conditions by circular dichroism. This took into account the reported behavior of LL-37, which assumes a helical conformation in the presence of biological membranes, but also in the presence of biologically relevant ions, in conditions that mimic human plasma (12, 31) (Fig. 5 and Table 2). Consistent with the literature, the presence of physiologically relevant ions induced structuring of LL-37 also in aqueous solution, whereas it did not structure at low pH,
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TABLE 1
Synthesized HDPs and their physico-chemical properties

| Peptide   | Sequence                                  | q<sup>1</sup> | ΦH<sup>2</sup> | μH<sub>rel</sub><sup>3</sup> | PIL<sup>4</sup> | SDS | TFE |
|-----------|-------------------------------------------|--------------|--------------|------------------|--------------|-----|-----|
| hssLL-37  | LLGDFRRSKKEIKGKEFKIVQR1KDFRNLVPRTES       | +6           | -1.83        | 0.59             | 52           | 58  | 57  |
| ppyLL-37  | LLGDFRRKAREK1GKEFKIVQR1KDFRNLVPRTES       | +4           | -1.70        | 0.59             | 32           | 58  | 54  |
| hmdSL-37  | SLGNFRRKARKK1GKEFKIVQR1KFKLHIFRTA         | +6           | -1.69        | 0.60             | 54           | 58  | 53  |
| mmuRL-37  | RGNFRRK1KK1GGGLKVK1QIKDFNLVPRTAS          | +8           | -1.66        | 0.56             | 7            | 60  | 57  |
| pobRL-37  | RGNFRRK1KK1GGGLKVK1QIKDFNLVPRTES          | +10          | -2.12        | 0.62             | <5           | 66  | 56  |
| cjaRL-37  | RLGDNQ1KQKEK1IGGLK1QIKDFGKAPRTES          | +5           | -1.94        | 0.61             | 42           | 48  | 42  |

<sup>1</sup> q, the charge.

<sup>2</sup> ΦH, mean residue hydrophobicity.

<sup>3</sup> μH<sub>rel</sub>, relative amphipathicity.

<sup>4</sup> the composition of PIL buffer is described in Table 2.

FIGURE 5. CD spectra of LL-37 and its primate homologues. A, LL-37: 1, 40 μM in 140 mM NaCl, pH 2.0; 2, 40 μM in SPB (10 mM sodium phosphate buffer, pH 7.0); 3, 10 μM in PBS (140 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0); 4, 100 μM in PBS; 5, 20 μM in PIL (113 mM NaCl, 24 mM NaHCO<sub>3</sub>, 0.6 mM MgCl<sub>2</sub>, 1.3 mM CaCl<sub>2</sub>, 3.9 mM KCl); 6, 40 μM in PIL; B, primate homologues in PBS (10 μM peptide); C, in PBS (100 μM peptide); D, in PIL (20 μM peptide); E, in 50% (v/v) TFE (20 μM peptide); F, in 10 mM SDS (20 μM peptide); hssLL-37 (—); ppyLL-37 (---); hmdSL-37 (-----); cjaRL-37 (—); pobRL-37 (-----); mmuRL-37 (——).
pointing to a stabilizing role of intramolecular electrostatic interactions (salt bridges) between anionic and cationic residues that are present in the peptide (Fig. 5A) (12). Structuring is accompanied by aggregation, as indicated by the concentration dependence of the helical content, likely leading to the formation of bundles via interaction of the hydrophobic faces on the helices (31). In the presence of TFE or SDS micelles (Fig. 5, E and F), the hydrophobic face can instead interact with a lipophilic environment, leading to the highest helical content, which was estimated from the ellipticity to be ~60% for LL-37 in both media, by the method of Chen et al. (24). Assuming that peptides in bulk solution are in equilibrium between unaggregated/random coil structures and aggregated/helical structures, and that aggregation leads to a similar maximum helical content as a lipophilic environment, then one can estimate the % aggregation under different aqueous solution conditions from the CD spectra, as reported in Table 2.

Having defined the condition-dependent behavior of hssLL-37, primate homologues were tested under the same conditions (Table 2 and Fig. 5). In PBS or PIL buffer the hmdSL-37, ppyLL-37, and cjaRL-37 structures and likely aggregate in bulk solution, in a similar manner to hssLL-37, whereas mmuRL-37 and pobRL-37 remain unstructured. In the presence of TFE or SDS micelles, however, all of the peptides show a similar maximum helical content (60–70%). Significantly, considering the distribution of basic and acidic residues in the polar sector of the helix, the tendency to structure in aqueous solution is observed only in those peptides that have an excess of electrostatic attractions over repulsions between residues separated by i + 3 or i + 4 positions from each other (Fig. 6) (12). This confirms that structuring is favored by the formation of intramolecular salt-bridges between cationic and anionic side chains in the peptides.

**Biological Activity of Primate Cathelicidins**—Given the different condition-dependent tendencies of the synthesized peptides to structure/aggregate, and the known sensitivity of the antimicrobial activity of hssLL-37 to the medium used in assays (11, 12), a series of different conditions were used also to test its antimicrobial properties (see Table 3). It was most active at the lowest salt and medium concentration conditions at which the microbes are still capable of growing (e.g. 5% (v/v) TSB or Sabouraud/dextrose medium in SPB (32, 33)). For Gram-negative bacteria, it retained activity up to 20% (v/v) TSB in SPB, but not in 20% (v/v) TSB in PIL buffer nor in 50% (v/v) MH broth, so that salt and medium both act to reduce activity. Conversely, for *S. aureus* hssLL-37 was active in 20% (v/v) TSB in PIL buffer but not in 50% (v/v) MH broth (data not shown). Results were substantially similar for hmdSL-37, whereas ppyLL-37 and cjaRL-37 both showed a marked general reduction in activity, possibly correlated with their lower charge. mmuRL-37 and pobRL-37, which are unstructured in bulk solution, were both active against all tested bacteria under all conditions, including 50% (v/v) MH. The highly charged pobRL-37 was also quite active against *C. albicans* in 20% (v/v) Sabouraud/dextrose medium.

**α-Helical antimicrobial peptides** generally act via a membrane-permeabilizing mechanism, so we tested the capacity of the primate LL-37 homologues to permeabilize bacterial membranes by monitoring the kinetics of hydrolysis of impermeant, extracellular, chromogenic substrates by periplasmic or cytoplasmic bacterial hydrolases. Results are summarized in Table 3 (see supplemental Fig. S2 for the hydrolysis curves in the presence of *E. coli* ML-35 pYC and *S. aureus* 710A). All peptides efficiently permeabilized both the outer and cytoplasmic membranes of *E. coli* in the absence of medium, but only mmuRL-37 and pobRL-37 did so in its presence. Permeabilization of the cytoplasmic membrane of *S. aureus* was considerably slower, and only mmuRL-37 and pobRL-37 showed an appreciable effect in the time scale of the experiment.

The kinetics of bacterial killing of representative Gram-positive and Gram-negative bacteria, exposed to selected peptides in PBS buffer before plating, are shown in Fig. 7. mmuRL-37 and pobRL-37 killed *E. coli* very efficiently under these conditions (>5 log drop in 10 min) and *S. aureus* more slowly (3–4 log drop in 30 min). This behavior is in line with previous observations on numerous model helical antimicrobial peptides (28, 34, 35). hssLL-37 was less efficient at killing *E. coli*, but surprisingly it was quite efficient at killing *S. aureus* under these conditions. This confirms the importance of medium effects in

### Table 2

| Conditions | Peptide concentration | Helix | Aggregation |
|------------|-----------------------|-------|-------------|
| 5 mM Na2HPO4/NaH2PO4, pH 7.0 (SPB) | 140 mM NaCl, 10 mM HCl, pH 2.0 | 140 mM NaCl in SPB, pH 7.0 (PBS) | 140 mM NaCl in SPB, pH 7.0 (PBS) | 113 mM NaCl, 24 mM NaHCO3, 0.6 mM MgCl2, 1.3 mM CaCl2, 3.9 mM KCl, pH 7.3 (PIL) | 10 mM SDS in SPB | 50% TFE in SPB | 10 mM SDS in SPB |
| 40 | 5 | 0 |
| 40 | 5 | 0 |
| 100 | 47 | 80 |
| 20 | 23 | 80 |
| 40 | 46 | 90 |
| 40 | 58 |
| 40 | 57 |

*Estimated assuming a maximum helical content in the presence of SDS.*

*Estimated at 222 nm by the method of Chen (24).*

**FIGURE 6. Pattern of repulsions or attractions in LL-37 and its primate homologues.** In the helical net projection of LL-37 shown in the left panel, i + 3 and i + 4 repulsions are indicated by a dotted line, whereas attractions are indicated by a solid line (after Ref. 12). A plot of the number of repulsions versus attractions for LL-37 and its primate homologues is shown in the right panel.
modulating its antimicrobial activity, as its killing activity in the presence of medium is markedly reduced (data not shown).

Finally, the capacity of the cathelicidin HDPs to lyse blood cells was determined and is reported in Table 3. At 10 μM peptides in PBS, hssLL-37 and hmdSL-37 were the most cytotoxic, whereas cjARL-37 was effectively inactive. At 100 μM peptide the pattern changes with mmuRL-37, hmdSL-37, and ppyLL-37 becoming highly cytotoxic.

The effect on membrane integrity and morphology of human lymphocytes, as assessed by flow cytometry, was then determined for hssLL-37 and mmuRL-37, respectively, taken as representative of aggregating and non-aggregating peptides. At an intermediate concentration of 40 μM, significant permeabilization of the membrane to PI was observed for hssLL-37 (42.9 ± 3.2% PI positive with respect to 11.4 ± 0.6% in the control, p < 0.001), whereas it was negligible for mmuRL-37 (11.7 ± 0.4% PI positive). Furthermore, the hssLL-37-induced membrane damage correlated with a significant morphological alteration in the cells, as indicated by a marked shift in side scatter (to 63.7 ± 2.1 mean channel from 51.3 ± 0.6 in the control, p < 0.001), whereas this was less evident in cells exposed to the macaque peptide (55.2 ± 1.5 mean channel).

Gene Sequence Analyses—The complete nucleotide sequences of the CAMP coding region for two or more individuals from each of 20 primate species were determined. The aligned sequences are provided in supplemental Fig. S1. When necessary, the consensus for each species was first obtained by alignment of the sequences of each individual per species to take intraspecific polymorphisms and variability into account (see below).

The CAMP gene showed a considerable nucleotide similarity between human and the Great Apes, including the last exon, which carries the HDP sequence. A greater variability was observed with the other non-human primate species, although the degree of conservation remains relatively high except in exon four, which is the least conserved. Nucleotide sequence variations well reflect the phylogenetic distances within primates species and are group specific.

To evaluate if selective pressures are acting differently on the different domains of the cathelicidin molecule, the sequences of the primate CAMP genes were subdivided into the three regions: signal, pro-region, and mature HDP. These were then used to compute Neighbor Joining trees with the all primate species studied. A maximum-likelihood approach, using the PAML suite of programs, was then used to identify, first, if there were residues that have been subject to positive selection in primates; and then, on what lineage this selective pressure acted. The Neighbor Joining tree for the mature HDP region is shown in Fig. 8, and closely reflects the expected phylogenetic relationship both among families and between families.

These analysis found that the whole signal sequence and pro-region (the cathelin-like domain) evolved under purifying or neutral selection (ω ≈ 1). On the contrary, the region encoding the mature HDP presented 71% of codons having evolved under positive selection, with a high average ω (ω = 6). Of these, several residues are identified as being under positive selection with high confidence (posterior probability greater than 0.99: Leu-1, Asp-4, Lys-10, Glu-11, Gly-14, Lys-15; posterior probability greater than 0.95: Phe-5, Ser-9, Phe-17, Ile-20, and Val-21). This is an indication that regions in the antimicrobially active portion of primate CAP-18, homologous to human LL-37, has been subject to positive selection (see supplemental Fig. S3).

Following the branch-analysis approach, all branches were found to be under selective pressure (ω > 1). No significant differences among the ω of the primate lineages, even if branches a (NWM-others, see Fig. 8) and b (Cercopithecidae-Hominidae) seem to be the longest, considering their t (nucleotide substitution per codon) (data not shown).
As we have analyzed multiple subjects from each species, albeit a limited set, we also looked for intraspecific variability and the possible presence of genetic polymorphisms. We found only one example, which is classifiable as a cSNP, represented by a Gly to Ala transition (originating a Gly/Glu change) at the second base in codon 162 in one of the three analyzed Cebus capucinus (two individuals were homozygous G/G, whereas the third heterozygous G/A). We assumed the G allele as the representative of the species as this position is conserved within the NWM.

DISCUSSION

In the primate cathelicidins, the gene region coding for the putative host defense peptides homologous to the human LL-37 has been subject to significant variation, with strong evidence for positive selection. The pro-region (cathelin-like domain) is more conserved (Figs. 1, 2, and supplemental S1), as also observed in other mammalian species (2). Variation in the primate HDP occurs at specific residues (Figs. 2, 3, and supplemental S3), markedly affecting the net charge while little affecting the overall hydrophobicity and amphipathicity. Furthermore, conserved residues seem to cluster in regions that are also maintained in homologous peptides from rodents, dog, and bovids (Fig. 4), possibly indicating a conserved structural or functional role for these stretches. In this respect, it is interesting that residues estimated to be under positive selection with a high confidence (posterior probability >0.95) cluster in the N-terminal half (up to residue 21), whereas those that are conserved in both primates and other mammals tend to cluster in the C-terminal half.

Six of the primate cathelicidin HDPs, including the human one, were selected for synthesis and characterizing, using criteria of maximum molecular and taxonomic diversity. Structuring was studied by circular dichroism under several conditions, and a striking variation in their behavior was observed. The more cationic pobRL-37 and mnuRL-37, which have an excess of intramolecular electrostatic repulsions over attractions (Fig. 6), were unstructured in bulk solution, irrespective of their concentration or that of monovalent or divalent cations and anions (Fig. 5). Conversely, the human and orangutan LL-37, the gibbon hmdSL-37 and NWM cjaRL-37, structured and likely aggregated to different extents in a salt-dependent manner (Table 2 and Fig. 5). All peptides, however, assumed a stable, and likely unaggregated helical conformation in the anisotropic environment provided by TFE or SDS micelles.

From our characterization of structuring and antimicrobial activity in vitro, a model for their action is proposed as illustrated in Fig. 9. Peptides of the LL-37 group seem to have evolved features that, under physiological conditions, tilt the equilibrium toward an aggregated “A-form,” rather than a free, monomeric, random coil “F-form.” This may enhance their capacity to interact with some types of biological membranes (Table 3 and Figs. 7 and supplemental S2), so affecting both antimicrobial and cytotoxic activities (31). It also seems to make the peptides rather “sticky,” so that they strongly interact with medium components in a manner that sequesters them (“S-form”), considerably reducing activity in full medium (11) and this may also explain the lowering of the LL-37 antimicrobial activity in serum (36) or lung surfactant (37). It could also provide a means for bacteria to develop resistance to the direct antimicrobial activity of the peptides, via secretion of exopolysaccharides (38). Peptides of the cercopithecinae RL-37 group instead exist exclusively in the F-form in bulk solution, having both a low tendency to structure/aggregate in solution.
Evolution of the Primate Cathelicidin

FIGURE 9. Schematic representation of the various structural forms for primate cathelicidin HDPs. Some peptides are in equilibrium between an unstructured free (F) form and a structured aggregated (A) form. These peptides can also bind to medium components, which segregate them and reduce activity (S form). All peptides interact and structure on the membrane surface (M form). Polar and hydrophobic faces are indicated in black and white, respectively.

or to bind to medium components. All peptides, however, seem to behave similarly in a lipophilic environment so can switch to a membrane inserted form (M-form) on contact with biological membranes. It is as yet unclear whether the predominant form in bulk solution (F, A, or S) affects only the approach to the membrane and the insertion step, then leading to substantially similar M-forms, or if it also affects the peptides interaction with the bilayer itself, leading to different M-forms.

We have been able to reproduce this behavior also in short (19 residue) model helical peptides (28) that have similar charge (+5) and hydrophobicity/amphipathicity characteristics to hssLL-37. By appropriate use of non-proteinogenic amino acids, we have been able to induce stable structuring and aggregation in one peptide (A-form), and have observed how this induces a strong medium dependence to its in vitro antimicrobial activity (S-form), which is absent in quite similar peptides that do not structure in bulk solution (F-form). Furthermore, the stabilized 19-mer (like LL-37) was more hemolytic and in flow cytometric analyses induced a greater permeabilization of lymphocytes to PI than the F-form, accompanied by increased side scattering. This indicates that the two types of peptides act via distinct mechanisms that depend on macroscopic structural characteristics (i.e., the adopted structural form in bulk solution), rather than specific features of the size, sequence, or charge distribution. For LL-37, the correlation between permeabilization to PI and increased side scatter has been reported also for monocytes, and it is apparently an energy-dependent process (39). It is an indication that, at least for host cells, there may be different M-forms for the two types of peptides.

The question remains as to why the analyzed primate cathelicidin HDPs have evolved in the observed manner. At first sight, it would seem that natural selection has favored a potent, salt- and medium-insensitive direct antibiotic function in some species (e.g. macaque and presbytis), but not in others (e.g. orangutan and capuchin monkey). However, the issue is complex, and the in vitro assays normally used to assess these activities may be misleading. It is, furthermore, feasible that the specific structural features observed in the single peptides may have been dictated also by other defense functions, such as immunomodulation or healing, so that the evolution of the peptides may have affected the balance between direct and indirect antimicrobial activities in a different manner in different primate species, depending on the different types of microbial biotas and infections to which they are exposed. Furthermore, it has been reported that epithelial LL-37 can be processed by serine proteases and the shortened peptides gain in antimicrobial activity (40, 41), possibly because intramolecular salt-bridging is reduced, allowing the fragments to switch to the F-form. Thus, a given peptide may have evolved so as to present different features in the complete and processed forms. In this respect, it is significant that the reported core region necessary for antimicrobial activity (residues Val-21 to Asn-30) (41) coincides with a highly conserved stretch in all mammalian orthologues of LL-37 (see Fig. 4). It is tempting to speculate that the peptides may have two or more domains with distinct functions, with the N-terminal stretch being subject to rapid evolution under positive selection, and the more conserved C-terminal stretch having a function that may be maintained in primate and non-primate mammalian species.

One could conjecture that the observed variations are related to differences in lifestyle, geographic distribution, or feeding habits, which determine the types of microbial biotas to which each primate species is exposed, and which are characterized by a great heterogeneity (42, 43). Humans, chimpanzees, baboons, macaques, and vervet monkeys, for example, tend to have “totipotent” feeding habits (occasionally or frequently omnivorous), whereas other species have more vegetarian diets (e.g. orangutan and gorilla). The two presbytis species analyzed have a quite specialized feeding behavior, mainly folivorous, and sacculated stomachs that support cellulose-digesting bacteria. Considering cationicity as an evident positively selected characteristic, the highest values (+11/+10) are encountered in the CAE and POB peptides, respectively, from an invertebrate omnivore (the vervet monkey) and a specialized herbivore (the silver crested monkey), whereas the lowest values (+4/+5) are encountered in the PPY and CJA peptides, respectively, from frugivorous orangutan and sap-eating marmoset, which both include insects and eggs in their diet. Thus, the general lifestyle characteristics described above are insufficient to provide any insight into the evolution of these peptides.

In conclusion, we have found that primate cathelicidin HDPs have been subject to selective pressure for variation, with evidence for positive selection throughout the phylogenetic tree relating the peptides, which favors alterations in the charge while little affecting overall hydrophobicity or amphipathicity. This variation apparently results in two distinct types of behavior. The first is similar to that of “canonical” helical antimicrobial peptides found in many insect (e.g. cecropins) and frog (e.g. magainins) species (6), and as cathelicidins in other mammalian species (e.g. SMAP-29 or BMAP-27 and -28) (7), and consists of a potent, salt- and medium-insensitive antimicrobial activity in vitro, which is possibly the principal function also in vivo.
The second type of behavior, as shown also by the human peptide, derives from a salt-dependent structuring in bulk solution that affects antimicrobial and cytotoxic activity, and makes it more medium-sensitive. Given the numerous reported immunomodulatory activities of hssLL-37, it is tempting to speculate that this behavior may also affect its indirect antimicrobial functions, which may be as or more important than the direct antibiotic activity in the full-length peptide. These considerations underline the importance of studying the effects of evolutionary variation in homologous HDPs from closely related species on a broad spectrum of activities related to host defense.

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