The human anaphylatoxin peptide C3a, generated during complement activation, exerts antimicrobial effects. Phylogenetic analysis, sequence analyses, and structural modeling studies paired with antimicrobial assays of peptides from known C3a sequences showed that, in particular in vertebrate C3a, crucial structural determinants governing antimicrobial activity have been conserved during the evolution of C3a. Thus, regions of the ancient C3a from Carcinoscorpius rotundicauda as well as corresponding parts of human C3a exhibited helical structures upon binding to bacterial lipopolysaccharide permeabilized liposomes and were antimicrobial against Gram-negative and Gram-positive bacteria. Human C3a and C4a (but not C5a) were antimicrobial, in concert with the separate evolutionary development of the chemotactic C5a. Thus, the results demonstrate that, notwithstanding a significant sequence variation, functional and structural constraints imposed on C3a during evolution have preserved critical properties governing antimicrobial activity.

In vertebrates, the complement system is activated by the classical, alternative, and lectin pathways, each converging at the step of complement factor 3 (C3)2 with release of multiple proteolytic fragments, including the anaphylatoxin C3a (1). In addition to its multiple proinflammatory functions involving histamine release from mast cells, smooth muscle contraction, and increased vascular permeability (1), C3a exerts a direct and potent antimicrobial effect against Gram-negative and Gram-positive bacteria (2). C3 deficiency is connected with increased susceptibility to bacterial infections in humans (3) as well as in animal models (4, 5), findings compatible with this direct antibacterial effect of C3a (5). Thus, C3a, in concert with other antimicrobial peptides (AMPs), executes pivotal roles in the innate immune system, providing a rapid and nonspecific response against potentially invasive pathogenic microorganisms (6). It has been demonstrated that cathelicidins and defensins (for review, see Refs. 6–8) representing two important AMP families display a wide sequence heterogeneity, thus reflecting positive selection and an adaptation of organisms to various bacterial environments (9–11). The complement factor C3 represents an evolutionarily old molecule (12) identified in the deuterostome Ciona intestinalis (13) as well as in the horseshoe crab Carcinoscorpius rotundicauda, a protostome considered a “living fossil” originating over 500 million years ago (14). These animals, which lack adaptive immunity, mount an effective antimicrobial defense in response to pathogens. In this work utilizing a combination of phylogenetic studies and structural and sequence alignments paired with biophysical and functional analyses, we have shown that structural prerequisites governing antimicrobial activity can be traced from the human C3a molecule back to the C3a molecules of invertebrates, such as those found in C. rotundicauda.

EXPERIMENTAL PROCEDURES

Microorganisms—Escherichia coli 37 A, Enterococcus faecalis 2374, Pseudomonas aeruginosa 27.1, E. coli American Type Culture Collection (ATCC) 25922, Bacillus subtilis ATCC 6633, and Candida albicans ATCC 90028 were obtained from the Department of Microbiology, Lund University, Lund, Sweden. Peptides and Proteins—C3a and C4a were obtained from The Binding Site, Inc. (San Diego, CA), whereas C5a-desArg was from Calbiochem. The peptides GKE31, LGE33, CNY21, CQF20, CVF20 (for sequences, see Fig. 2), and tetramethylrhodamine (TAMRA)-conjugated CNY21 and CQF20 were synthesized by Innovagen (Lund, Sweden). The purity (>95%) and molecular weight of these peptides were confirmed by mass spectral analysis (matrix-assisted laser desorption ionization time-of-flight, Voyager). 20-mer peptides corresponding to various regions of C3a (Fig. 2 and supplemental Table 2) were from Sigma (PEPScreen®, Custom Peptide Libraries, SigmaGenosys).

Phylogenetic Analyses—C3a, C4a, and C5a amino acid sequences were retrieved from the NCBI site. Each sequence was analyzed with Psi-Blast (NCBI) (15) to find the ortholog and paralog sequences. Sequences that showed structural homology >70% were selected. These sequences were aligned...
using ClustalW (16) using Blosum 69 protein weight matrix settings (17). Internal adjustments were made taking the structural alignment into account utilizing the ClustalW interface. The level of consistency of each position within the alignment was estimated by using the alignment-evaluating software Tcoffee (18). C3a, C4a, and C5a sequences were used for phylogenetic tree construction by using the neighbor-joining method (19). The generated tree was rooted with human C3a, and the reliability of each branch was assessed using 1000 bootstrap replications. For clarity, amino acid numbering in the text below is based on the position in the respective anaphylatoxin peptide, defining the N terminus after the RKKR processing site. Structural alignment of C3a and C5a structures revealed that the regions corresponding to Arg8–Tyr15, Leu19–Met27, and Lys50–Leu63 in human C3a (Fig. 2) correspond to structure-conserved regions. Residues Ile2–Asn67 of C. rotundicauda C3a were built using the Prime module (22) from the Schrödinger computational chemistry suite of programs (Schrödinger, L.L.C., Portland, OR). The sequence identity was 26% (similarity 38%), and rotamers from the conserved residues were retained. Terminal tails beyond secondary structure elements were not built. Loops 1 (Glu11–Arg13) and 2 (Asp27–Arg29) were refined one at a time using default sampling in the loop refinement protocol built into Prime, except the long inserted loop 3 (Glu39–Glu47) where extended medium sampling was used. Disulfur bridges Cys17–Cys49, Cys18–Cys56, and Cys31–Cys57 were built and minimized prior to refinement. A stepwise refinement protocol involving minimization of (a) side chains from non-structure-conserved regions, (b) both backbone and side chains from non-structure-conserved regions, and (c) all side chains were employed. The refined comparative model of C. rotundicauda C3a was extended in the C terminus by residues Ile68–Arg75. These residues were added in an α-helical conformation and minimized. Some adjustment of the ψ/φ values of Leu64, Leu65, Lys66, and Asn67 to helical values were necessary prior to minimization. All atoms of residues Leu64–Arg75 were minimized of this extended model. Finally, all side chains were minimized resulting in the described model. Minimization was performed using the MacroModel module from the Schrödinger computational chemistry suite of programs using a dielectric constant of 1 and the OPLS2005 force field. Comparative homology models of human C4a (residues 1–77) and C. intestinalis C3a (residues 2–76) were constructed using the same template, refinement protocol, and minimization as above and the alignment in Fig. 2. The last seven and five resi-
dues, respectively, were added in α-helical conformations and minimized. Sequence identities were 29% (51% similarity) for human C4a and 17% (37% similarity) for C. intestinalis C3a when compared with human C3a.

Radial Diffusion Assay—Essentially as described earlier (23), bacteria were grown to mid-log phase in 10 ml of full-strength (3% w/v) trypticase soy broth (TSB) (BD Biosciences). The microorganisms were washed once with 10 mM Tris, pH 7.4.

4×10^6 bacterial colony-forming units were added to 5 ml of the underlay-agarose gel (0.03% TSB, 1% low electroendosmosis-type agarose (Sigma) and 0.02% Tween 20 (Sigma)). The underlay was poured into an 85-mm-diameter Petri dish (or 144 mm diameter for the experiment with PEPscreen peptides). After agarose solidification, 4-mm-diameter wells were punched, and 6 μl of test peptide was added to each well. Plates were incubated at 37 °C for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 5 ml of molten overlay (6% TSB and 1% low electroendosmosis-type agarose in distilled H_2O). The antimicrobial activity of a peptide was visualized as a zone of clearing around each well after incubating 18–24 h at 37 °C. The activity of the human complement-derived peptides LGE33, CNY21, CQF21, and CVV20 as well as GKE31 of C. rotundicauda were compared with the activity of the peptide LL-37. In all cases, triplicate samples were used.

Viable Count Analysis—P. aeruginosa 27.1 or E. faecalis 2374 bacteria were grown to mid-log phase in Todd–Hewitt medium. Bacteria were washed and diluted in 10 ml Tris, pH 7.4, containing 5 mM glucose. For dose-response experiments, P. aeruginosa (50 μl; 2×10^6 colony-forming units/ml) were incubated at 37 °C for 2 h with the peptides GKE31 and LGE33 (P. aeruginosa) at concentrations ranging from 0.03 to 60 μM. For analysis of the effects of C3a, C4a, and C5a-desArg (see Fig. 7A), P. aeruginosa and E. faecalis 2374 were used, and the peptide concentration was 3 μM. To quantify the bactericidal activity, serial dilutions of the incubation mixture were plated on Todd–Hewitt agar followed by incubation at 37 °C overnight, and the number of colony-forming units was determined.

Electron Microscopy—P. aeruginosa 27.1 (1–2×10^7/sample) were incubated for 2 h at 37 °C with the peptides GKE31 or LGE33 at ~90% of their required bactericidal concentration (6 μM), as judged by dose-response experiments using viable count assays (not shown). LL-37 (6 μM) was included as a control. Samples of P. aeruginosa bacteria suspensions were adsorbed onto carbon-coated copper grids for 2 min, washed briefly on two drops of water, and negatively stained on two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in ambient air. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 60 kV of accelerating voltage. Images
Circular Dichroism (CD) Spectroscopy—The CD spectra of the peptides in solution were measured on a Jasco J-810 spectropolarimeter (Jasco). The measurements were performed at 37 °C in a 10-mm quartz cuvette under stirring, and the effect on peptide secondary structure was monitored in the range of 200–250 nm. The background value (detected at 250 nm, where no peptide signal is present) was subtracted, and signals from the bulk solution were corrected. The peptide secondary structure was monitored at a peptide concentration of 10 μM both in Tris buffer and in the presence of E. coli lipopolysaccharide (0.02% w/w) (E. coli 0111:B4, highly purified, <1% protein/RNA; Sigma).

Liposome Preparation and Leakage Assay—Dry lipid films were prepared by dissolving either dioleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) (60 mol %) and cholesterol (Sigma, St Louis, MO) (40 mol %) or dioleoylphosphatidylcholine (30 mol %), dioleoylphosphatidic acid (Avanti Polar Lipids, Alabaster, AL) (30 mol %), and cholesterol (40 mol %) in chloroform and then removing the solvent by evaporation under vacuum overnight. Subsequently, buffer (10 mM Tris, pH 7.4) was added together with 0.1% carboxyfluorescein (CF) (Sigma). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles consisting of freezing in liquid nitrogen and heating to 60 °C. Unilamellar liposomes of ~100 nm diameter were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a Lipofast minixertruder (Avestin, Ottawa, Canada) at 22 °C. Untrapped CF was then removed by two gel filtrations (Sephadex G-50) at 22 °C with the Tris buffer as eluent. The CF release was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10 mM lipid in 10 mM Tris, pH 7.4). An absolute leakage scale was obtained by disrupting the liposomes at the end of the experiment through the addition of 0.8 mM Triton X-100 (Sigma), thereby causing 100% release and dequenching of CF. A SPEX-fluorolog 1650 0.22 monochromatic double spectrometer (SPEX Industries, Edison, NJ) was used for the liposome leakage assay.

Fluorescence Microscopy—P. aeruginosa 27.1 bacteria were grown to mid-logarithmic phase in Todd-Hewitt medium. The bacteria were washed twice in 10 mM Tris, pH 7.4. The pellet was dissolved to yield a suspension of 5 × 10^8 colony-forming units/ml in the same buffer. Two hundred microliters of the bacterial suspension were incubated with either 1 μl of TAMRA-CNY21 or TAMRA-CQF21 (2 mg/ml) on ice for 5 min and washed twice in 10 mM Tris, pH 7.4. The bacteria were then fixed by incubation on ice for 15 min and in room temperature for 45 min in 4% paraformaldehyde. The suspension was applied onto poly-L-lysine-coated cover glass, and bacteria were left to attach for 30 min. The liquid was poured away, and the cover glass was mounted on a slide by Dako mounting medium (Carpinteria, CA). This was performed using a Nikon
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Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charge-coupled device camera, a Plan Apochromat 60× objective, and a high numerical aperture oil condenser.

RESULTS

Sequence Analyses of Anaphylatoxin Homologs—Phylogenetic analysis of representative C3a peptides from invertebrates as well as vertebrates (Fig. 1) yielded valuable information. Consistent with a common ancestor, the C3a molecules all colocalized to a single group, suggesting that C3a has evolved via multiple changes in the protogene, a finding consistent with previous analyses of the evolution of the complement system (14, 24). On the other hand, C4a and C5a formed separate clades, C5a being most distant from the C3a. This pattern suggests that C5a and C4a likely evolved from C3a and that C5a is the paralog to C4a and C3a (14). Comparisons of synonymous and non-synonymous substitution rates are useful for studying the mechanisms of gene evolution. Analysis of evolutionarily distant sequences, however, is not useful because of the saturation of amino acid changes. Nevertheless, to get useful information on possible positive selection of C3a, relevant and homologous vertebrate sequences were compared. The dS values (indicating synonymous nucleotide substitutions) ranged from 0.7 to 1.0, except for Macropus eugenii, which showed a higher substitution rate ~2.7, whereas the dN (indicating non-synonymous substitutions) ranged from 0.1 to 0.2 (supplemental Table 1). Thus, although there was the existence of an extensive variability of certain amino acids in C3a (Fig. 2), the results from the analysis of vertebrate sequences indicated that the selection pressure imposed on the C3a molecules results in a high degree of conservation.

Given this phylogenetic relationship, it was of interest to examine this molecular family closer, both from a structural as well as a functional perspective. Several common structural features exist for the corresponding C3a, C4a, and C5a sequences of various organisms crucial for the integrity and stability of the molecules (Fig. 2). A most notable feature is the existence of six disulfide-bonded cysteines, which are conserved not only in C3a but also in C4a and C5a. The three disulfide bonds stabilize the conformation of the internal "core" portion of the molecules represented by residues 22–57 in the human C3a sequence. The C3a of C. intestinalis lacks one disulfide pair (Fig. 2) and will be discussed separately below. Focusing on C3a, it is evident that, apart from cysteines, the four glycines (positions 13, 26, 46, and 74), phenylalanine (53), as well as lysines and arginines (21, 64, 77) constitute additional conserved features, suggesting their importance for the structural stability as well as function of C3a.

Structural Modeling of Anaphylatoxin Peptides—Computational modeling utilizing available structural data on human C3a (21, 25) as well as C5a peptides (26, 27) was employed to provide further structural information. Considering the recent identification of C3 and a putative anaphylactic peptide in the arthropod C. rotundicauda (14), we decided to compare these two molecules. As demonstrated in Fig. 3A, the similarity at the three-dimensional level between human C3a and the predicted C. rotundicauda C3a peptide is apparent, although having an extensive overall sequence discrepancy (26% sequence identity and 38% similarity). Both peptides share a striking similarity in the four helical regions and in the two first loops located before the cysteines at positions 22 and 36 and positions 17 and 31 in the human and C. rotundicauda peptides, respectively. The C. rotundicauda C3a has a five-amino-acid-long insert in the third loop region between the cysteines at positions 31 and 49, a feature only shared with C3a from C. intestinalis. Analogous to human C3a, the C. rotundicauda peptide contains a prominent cationic and amphipathic helical C terminus predicted to extend a few residues further than in C3a.
human C3a. In human C3a, the C-terminal part of this region, being flexible in solution, strongly conforms to an \( \alpha \)-helical conformation in anisotropic environments (1). Helical wheel diagrams (Edmundson projection) illustrate the amphipathic nature of the C-terminal helices (given a helical conformation) (Fig. 3B), as defined by the peptides GKE31 and LGE33, which encompass this region of \( C. \ rotundicauda \) and human C3a, respectively (see Fig. 2 for sequence). The amphipathic organization of these helices is also seen in the three-dimensional models of \( C. \ rotundicauda \) and human C3a (Fig. 3A).

**Antimicrobial Activities of C3a Peptides**—To explore the structure-function relationships of C3a epitopes, overlapping peptide sequences comprising 20-mers (Fig. 2 and supplemental Table 2) were synthesized and screened for antimicrobial activities against \( P. \ aeruginosa \), a ubiquitous pathogen found among both vertebrates as well as invertebrates. The experiments showed that, particularly, peptides derived from the C-terminal regions of \( C. \ rotundicauda \), \( Ciona \), and \( Branchiosoma \) as well as from the vertebrates \( Homo \), \( Sus \), \( Mus \), \( Rattus \), and \( Guinea \), were antimicrobial (Fig. 4), whereas peptides originating from \( Cobra \), \( Paralichtys \), \( Onchorhyncus \), \( Eptatretus \), and \( Xenopus \), and \( Gallus \) did not show any activity against bacteria (Fig. 4). Properties common for most AMPs include minimum levels of cationicity, amphipathicity, and hydrophobicity (6). Therefore, it was interesting to note that C-terminal regions of the two former groups comprised cationic peptides, whereas the latter group comprised negatively charged peptides (illustrated by colors in Fig. 4). These results corresponded well with the phylogenetic analysis, which showed that these animals belong to different subclades, and with single nodes of divergence (Fig. 1). The global analysis of biophysical parameters showed that, in general, peptides displaying antimicrobial activity had a net charge of +2 to +4 and ~20–40% hydrophobic amino acids. The degree of amphipathicity, as judged by the relative hydrophobic moment (\( \mu \text{Hrel} \)), ranged from ~0.2 to 0.4, values comparable with those observed in many helical AMPs (28). Although intu-
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Itively apparent (Fig. 4), the results confirmed that net charge correlates to antimicrobial activity (supplemental Fig. 1). Considering these findings, it is notable that a disproportionate alteration of charge appears to characterize the evolution of β-defensins (9, 29). Analogous relationships were recently reported to apply to the evolution of primate cathelicidin, showing positive selection affecting charge while keeping hydrophobicity and amphipathicity fairly constant (11).

Structural and Functional Congruence of C Termini of C3a—In humans, peptides derived from the well defined C-terminal region of C3a exert antimicrobial effects. Furthermore, human neutrophilic enzymes release similar C3a-derived peptides exerting antimicrobial effects, proving the physiological importance of this helical and antimicrobial region of C3a (2). Considering this, the following experimental analyses focused on the C-terminal region of human C3a as well as the related peptide “ancestor” from C. rotundicauda (see Figs. 2 and 3B). Indeed, the experiments showed that the C. rotundicauda peptide GKE31, spanning the whole C-terminal part of C. rotundicauda C3a, exerted similar antibacterial effects as the human homolog LGE33 against both the Gram-negative P. aeruginosa and E. coli and the Gram-positive B. subtilis (Fig. 5A). The “classical” human cathelicidin LL-37 yielded similar effects as the two C3a-derived peptides. The C. rotundicauda peptide was not active against C. albicans. To examine whether the GKE31 and LGE33 peptides interact with and permeabilize bacterial plasma membranes, P. aeruginosa was incubated with each of the two peptides at concentrations yielding ~90% bacterial killing (6 μM) and analyzed by electron microscopy (Fig. 5B). Clear differences in the morphology of peptide-treated bacteria in comparison with the control were demonstrated by this approach. The peptides caused local perturbations and breaks along P. aeruginosa plasma membranes, and occasionally intracellular material was found extracellularly. These findings were similar to those seen after treatment with the antimicrobial human cathelicidin LL-37 (Fig. 5B). Furthermore, CD spectroscopy was used to study the structure and the organization of the GKE31 and LGE33 peptides in solution and upon interaction with E. coli lipopolysaccharide (LPS) (Fig. 5C). Neither GKE31 nor LGE33 adopted an ordered conformation in aqueous solution; however, the CD spectra revealed that a significant and almost identical structural change, largely indicating an induction of helicity, taking place in the presence of E. coli LPS (Fig. 5C). The remarkably similar profile of both peptides indicates that, despite a marked difference in primary sequence, the peptides structure themselves in a similar way in the presence of negatively charged LPS-rich bacterial membranes. Both of the peptides also induced leaking of liposomes, thus establishing their membrane-breaking activities (Fig. 5D). Kinetic analysis showed that ~80% of the maximum fluorescence was reached within ~200 s for both peptides (at 1 μM) (Fig. 5E). The results therefore indicate that the GKE31 and LGE33 peptides indeed function similar to most helical AMPs, such as LL-37 (6, 28), by interactions with LPS and most likely peptidoglycan at bacterial surfaces, leading to induction of an α-helical conformation, which in turn facilitates membrane interactions, membrane destabilization, and, finally, bacterial killing. The fact that the two C3a-derived peptides are separated by as much as over a half-billion years of evolutionary distance elegantly demonstrates the remarkable structural and functional conservation of this C-terminal peptide region.

Comparison of C3a Molecules of C. rotundicauda and C. intestinalis—The C3a molecule of Ciona is functionally active; it exerts chemotactic effects (13) and, as shown here, has a C-terminal antibacterial part (Fig. 4). However, in contrast to the other C3a molecules, C3a of Ciona lacks one disulfide bridge (Fig. 2). Despite this, and a sequence identity of only 17% (37% similarity) with human C3a, molecular modeling and conformational analysis show that Ciona C3a adopts a predicted conformation similar to other C3a molecules (Fig. 6A). Interestingly, the first missing cysteine in Ciona C3a is replaced by a glycine residue (Gly18), whereas the second cysteine is replaced by a glutamate residue (Glu57). Hypothetically, these changes could enable the second helix to approach and interact with the C-terminal helix by formation of a salt bridge between Glu57 and Lys22, thus compensating for the loss of a disulfide bond. These interactions, together with main structural constraints imposed by the remaining two disulfide pairs preserve the overall topology of Ciona C3a (Fig. 6B). Furthermore, it is notable that residues forming the inner core throughout the anaphylatoxin family (Gly21, Ile/Val39, and Phe54) are all conserved in Ciona C3a (Fig. 2). Taken together, these structural considerations, combined with the functional data, further underscore the conservation of C3a.

Structure and Activities of C4a and C5a—The phylogenetic analysis indicated that C5a evolved separately from the family of C3a as well as C4a molecules (Fig. 1). To address...
whether this also reflected a functional difference, we compared the antibacterial activities of human C3a with C4a, and C5a (the latter only available in desArg form) and their corresponding peptides from the C terminus. C3a and C4a both exerted antibacterial effects, whereas the C5a peptide was inactive (Fig. 7A). Corroborating results were obtained with the corresponding C-terminal peptides (Fig. 7B; for sequences, see Fig. 2). Although the C3a-and C4a-derived peptides (CNY21 and CQF21, respectively) killed the Gram-negative P. aeruginosa and E. coli, the Gram-positive B. subtilis, as well as the fungus C. albicans, the C5a peptide (CVV20) was inactive against these microbes. The fact that the C5a-derived peptide CVV20 contained a terminal arginine and that there is no difference in antimicrobial activity between C3a and C3adesArg as well as the corresponding C3a-derived peptides CNY21 and CNY20 (2) demonstrated that the terminal arginine is dispensable for antimicrobial activity of these peptides. Finally, the interaction between the antimicrobial peptides CNY21 (C3a) and CQF21 (C4a) and bacterial plasma membranes was examined by fluorescence microscopy. The peptides were labeled with the fluorescent dye TAMRA and incubated with P. aeruginosa. As seen in the three-dimensional models, the last ten residues in C5a form a short α-helix that is bent backwards through a short loop positioning the carboxyl terminal arginine (Arg74) in close proximity to arginine 62 (Fig. 3A), a feature that is believed to be important for receptor binding (27). Thus, C5a display a significantly different structure when compared with both C4a and C3a, lacking the typical C-terminal and antimicrobial protruding peptide (Fig. 3A), thus compatible with the experimental results presented herein. This observation, paired with the separate evolutionary development of C5a, indicate that this molecule has evolved separately in higher organisms into a purely chemotactic and highly spasmogenic molecule.

**DISCUSSION**

In conclusion, the combination of phylogenetic, structural, biophysical, and biological analyses point at a preservation of structures crucial for antimicrobial activity of C3a in invertebrates as well as vertebrate lineages. Although speculative, the difference noted in C3a of jawless fishes, likely abolishing the antimicrobial activity of C3a, may not only reflect the multifunctional role of C3a but could also indicate a gain of other functions during the separation from invertebrates in the ordovician period (500 million years ago). The observation that anaphylatoxins have unforeseen biological effects in fish is compatible with this hypothesis (30). As mentioned previously, significant changes in cationicity are observed in defensins as
well as cathelicidins, even in closely related species (such as primates) (11, 31). Furthermore, indicative of a strong non-neutral evolution, gene duplications and subsequent variations have led to further generation of different AMPs (29). For example, the C-terminal domains of cathelicidins may range from a dozen to over eighty residues forming cysteine-bridged hairpins (bovine bactenecin) and tryptophan-rich (bovine indolicidin), proline-rich (porcine PR-39), or α-helical molecules (human LL-37) (8). In this context, it is interesting to note that a unifying structural motif (γ-core) was recently revealed in many diverse AMPs, such as protegrins, defensins, and chemokines, thus indicating the existence of previously unprecedented higher level structural motifs that govern antimicrobial activity (6). Being multifunctional, also acting as immune modulators, the generation of many structurally diverse AMPs likely reflects an evolutionary adaptation to the microbial environment as well as introduction of novel biological functions in a given organism. Contrasting to this, C3a has maintained strikingly similar features governing antimicrobial activity, despite a significant primary sequence variation. Thus, in addition to the “innovative” generation of AMPs in nature, our findings on C3a illustrate an alternative concept where molecules are subjected to strong and precise selection forces aiming at maintaining a robust and structurally intact innate defense system.

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