θ-Defensins: Cyclic Antimicrobial Peptides Produced by Binary Ligation of Truncated α-Defensins

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Abstract: The first cyclic peptide discovered in animals is an antimicrobial octadecapeptide that is expressed in leukocytes of rhesus monkeys. The peptide, termed rhesus θ-defensin 1 (RTD-1) is the prototype of a new family of antimicrobial peptides, which like the previously characterized α- and β-defensin families, possesses broad spectrum microbicidal activities against bacteria, fungi, and protects mononuclear cells from infection by HIV-1. The cyclic θ-defensin structure is essential for a number of its antimicrobial properties, as demonstrated by the markedly reduced microbicidal activities of de-cyclized θ-defensin analogs. Genetic and biochemical experiments disclosed that the biosynthesis of RTD-1 results from the head-to-tail joining of two nine-amino acid peptides, each of which is donated by a separate precursor polypeptide, which are in fact C-terminally truncated pro-α-defensins. Alternate combinations of the two nonapeptides generate two additional macaque θ-defensins, RTD-2 and RTD-3. Humans do not express θ-defensin peptides, but mRNAs encoding at least two θ-defensins are expressed in human bone marrow. However, in each case the open reading frame is interrupted by a stop codon in the signal peptide-coding region. The mature θ-defensin peptide is a two-stranded β-sheet that, like the α- and β-defensins, is stabilized by three disulfides. However, the parallel orientation of the θ-defensin disulfide arrangement allows for substantial flexibility around its short axis. Unlike α- and β-defensins, RTD-1 lacks an amphiphilic topology. This may partially explain the unusual interaction between θ-defensins and phospholipid bilayers.

Keywords: Theta-defensins, macrocyclic, cyclic peptides, antimicrobial.

INTRODUCTION

Defensins are antimicrobial peptides expressed in diverse life forms that include plants, insects, birds, and mammals including humans [1-4]. Most evidence to date supports the notion that defensins are important mediators of innate immunity, protecting the host by direct killing of microbes that at least transiently colonize the host. However, newer studies indicate that mammalian defensins have a role in regulating acquired immunologic responses [5].

The first two families of mammalian defensins discovered were the α- and β-defensins (Fig. **1**). Peptides of both defensin families are without exception cationic molecules, and those peptides that have been isolated range in size from 29 to 42 amino acids. Distinct tridisulfide motifs distinguish the α- and β-defensin families, but despite the differing cysteine connectivities, the peptide folds are very similar [Fig. **1**; 6]. In addition, the precursor structures of the peptides differ substantially. Six α-defensins have been isolated from human tissues: HNP 1-4 from blood leukocytes, and HD-5 and HD-6 from intestinal Paneth cells.

While much of the evidence for the host defense role of defensins is indirect, two recent studies using mouse models offer convincing proof in this regard. In the first, Wilson *et al.* showed that targeted disruption of α-defensin processing in the mouse small intestine was accompanied by a 10-fold increase in sensitivity to Salmonella infection [7, 8]. The cells that express α-defensins in the small intestine, secretory Paneth cells, discharge defensins into the lumen by sensing prokaryotic antigens [7, 8]. More recently, Bevins and colleagues reported that transgenic expression in mice of the human intestinal β-defensin HD-5 conferred resistance to *Salmonella typhimurium* in these animals [9]. *In vitro* studies demonstrate that human β-defensins are also microbicidal, like α-defensins possessing antimicrobial activities for bacteria, fungi, and HIV.

A third family of defensins, termed θ-defensins (θ was selected to maintain the Greek symbology of the earlier defensin subfamilies and to reflect the covalent conformation of the new family) was discovered more recently. As described below, θ-defensins are cyclic molecules biosynthesized by a novel pathway from α-defensin-like precursors.

DISCOVERY OF θ-DEFENSINS.

An analysis of α-defensins expressed in rhesus macaques was undertaken in order to characterize this aspect of innate immunity in an animal that is widely used for modeling host-pathogen interactions in humans. Macaque neutrophils were found to express at least eight α-defensins, four of which, not surprisingly, are nearly sequence-identical to human orthologs; four additional neutrophil α-defensins were isolated, but these were quite different in their primary structures [10]. Intermixed with chromatographic fractions

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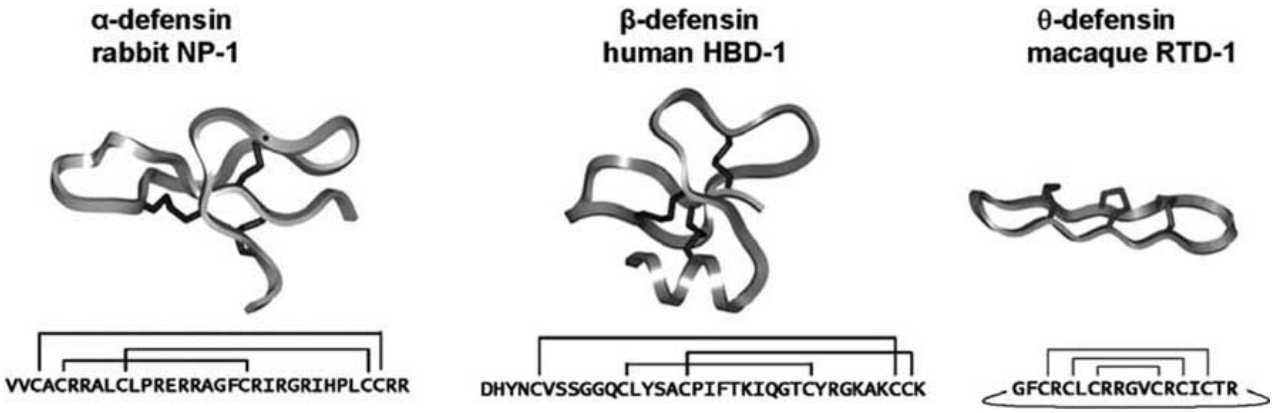

Fig. (1). Defensin peptides. Ribbon diagrams of representative members of the α, β, and θ-defensin peptide families are shown along with schematics of their covalent structures and disulfide bonding patterns.

containing the α-defensins was a peptide that was more active than any of the other peptides characterized. Amino acid analysis demonstrated that the peptide was rich in arginine and cyst(e)ine, but its molecular weight (2082.7 by mass spectroscopy) was far less than any α- or β-defensin. Sequence analysis revealed that the peptide, termed rhesus θ-defensin-1 (RTD-1) was a head-to-tail cyclized octadecapeptide, stabilized by three disulfide bonds [Fig. **1**; 11].

The RTD-1 structure was confirmed by solid phase synthesis [11]. Synthesis was facilitated by the fact that a linear form of RTD-1, produced by assembling a nearly symmetrical chain with two-residue overhanging ends, quantitatively formed the correct disulfides under gentle oxidizing conditions. Thus the amino- (Gly-1) and carboxyl-terminal (Arg-18) residues were conveniently juxtaposed, and the loop-closing peptide bond was readily formed with a carbodiimide-mediate coupling reaction [11]. Initial attempts to synthesize RTD-1 by standard Fmoc protocols gave poor results due to the propensity of the cysteine residues to racemize, possibly a result of their density along the peptide chain. The production of racemic mixtures was minimized by using the preformed pentafluorophenyl ester of cysteine, generating a product that was biologically and biochemically indistinguishable from natural RTD-1 [11]. Gram quantities of RTD-1 have now been prepared using this synthetic scheme, enabling numerous studies including peptide crystallization (Fig. **2**).

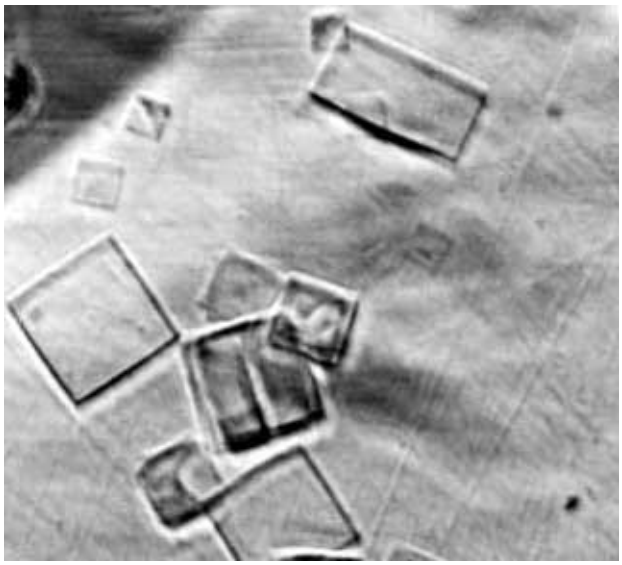

Fig. (2). RTD-1 crystals. Cubic crystals were grown as sitting drops from a solution of synthetic RTD-1 equilibrated with ammonium phosphate, pH 3.8.

GENE STRUCTURE AND EXPRESSION.

As with other cyclic polypeptides, elucidation of the RTD-1 gene structure was not straightforward, as the linear relationship between the peptide chain and the corresponding mRNA was unknown. In fact, we surmised that RTD-1 was probably a primate member of the cathelicidin family [12-14] because the peptide so strongly resembled the cathelicidin protegrin-3, a porcine leukocyte-derived, cationic antimicrobial peptide that contains two disulfides and multiple arginines [Fig. **3**; 14]. However, screening of a macaque bone marrow cDNA library with a cathelicidin-specific probe yielded no RTD-1-encoding sequences. An alternative approach was employed wherein macaque bone marrow cDNA was amplified by carrying out RT-PCR reactions employing oligonucleotide primers corresponding to sequential hexapeptide sequences within the RTD-1 backbone [11]. Two cDNAs were isolated, but surprisingly, neither of the two full length cDNAs encoded the entire 18-residue peptide. However, inspection of the sequences revealed that RTD-1 was composed of amino acid sequences derived from two gene-encoded precursors, RTD-1a and RTD-1b (Fig. **4**). RTD-1a/1b are myeloid α-defensin homologs, differing only by the fact that the open reading frame is prematurely truncated by a stop codon after three residues C-terminal of the third conserved α-defensin cysteine (Fig. **4**). The corresponding genes (RTD1.1 and RTD1.2) possess the prototypical 3-exon/2intron structure of all other known myeloid defensins, and their nucleotide sequences are 88% identical to that of an α-defensin-like pseudogene. Thus, it appeared that RTD-1 was composed of two 9-residue peptides, linked head-to-tail, which derive from the highly conserved RTD-1a/1b precursors.

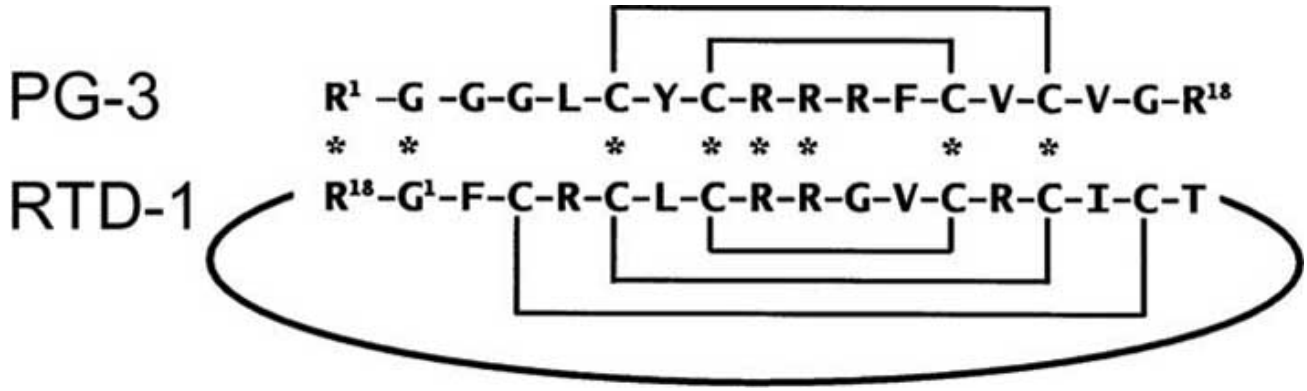

Fig. (3). Similar covalent structures of protegrin-3 and RTD-1. The sequences of protegrin-3 (PG-3), a cathelicidin, and RTD-1 were maximally aligned. Identical residues are marked with asterisks.

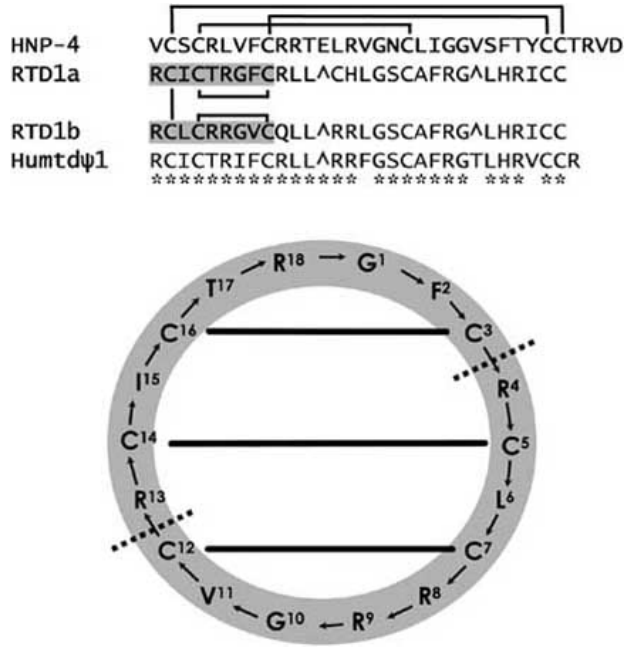

Fig. (4). θ-defensins are binary ligation products of truncated α-defensins. Top- Amino acid sequences and disulfide bonding patterns of an α-defensin (HNP-4), two pro-θ-defensins (RTD1a and RTD1b), and a human θ-defensin pseudogene (Humtdψ1) are aligned. Expressed amino acids are in bold text, whereas normal text denotes amino acids that would be encoded in the absence of termination codons [^]. Asterisks denote amino acid identity between RTD1a or RT1b and Humtdψ1. Shaded amino acids in the RTD1a and RTD1b sequences correspond to the respective nonapeptides that are ligated, head-to-tail, in the mature RTD-1 (bottom). The dotted lines in the RTD-1 schematic designate the positions of the new peptide bonds formed.

The post-translational processing steps required for producing mature RTD-1 include a) removal of the signal peptide, b) proteolytic cleavages at sites flanking each of the donor nonapeptides, and c) formation of two new peptide bonds (Fig. **5**). In addition, the cysteine connectivities must be properly arranged, though we expect that the disulfides are probably involved in orienting the two peptide chains (discussed further below). Of note is the fact that none of the disulfide connectivities of an α-defensin (e.g., HNP-4 in Fig. **4**) occur in RTD-1.

If the binary excision/ligation pathway proposed in Figure **5** is in fact required for the biosynthesis of RTD-1, one might expect that different pairings of the RTD1a & RTD1b-derived nonapeptides could generate distinct RTD-1 like θ-defensins. Indeed, RTD-2 (RTD1b/1b) and RTD-3 (RTD1a/1a) were isolated from rhesus macaque bone marrow [15] and peripheral blood leukocytes [16]. Interestingly, the abundance of RTD 1-3 in macaque leukocytes is approximately 30:1:2, respectively, demonstrating that the predominant θ-defensin in these cells is the heterodimeric conformer [16]. The alternative pairing of pro-θ-defensin-derived nonapeptides produces three θ-defensins that vary in their charges from +4 (RTD-3) to +6 (RTD-2). A third rhesus macaque pro-θ-defensin was predicted by cDNA cloning [17], potentially giving rise to three additional θ-defensins having net charges of +2, +3, or +4. Recent studies provide evidence for peptide expression of at least two of these predicted θ-defensins (Tran and Selsted, unpublished data).

In macaque peripheral blood, θ-defensin peptide is most abundant in neutrophils, and the peptide is stored in cytoplasmic granules [Fig. **6**; 11]. Neutrophil θ-defensin biosynthesis is complete by the time the cell exits the bone marrow, so while neutrophilic precursors are strongly positive for θ-defensin mRNA, blood neutrophils are devoid θ-defensin transcripts. Interestingly, unfractionated blood leukocytes express θ-defensin mRNAs. We have recently shown that these transcripts are produced by monocytes, cells shown previously to be immunopositive for RTD-1 [11]. This finding is consistent with another recent study demonstrating that α-defensins are expressed in human peripheral blood monocytes [18]. Lymphocytes, eosinophils, and platelets do not express defensins. Moreover, a Northern blot survey of rhesus macaque tissues indicated that bone marrow was the only site of θ-defensin expression in adult animals [11].

ANTIMICROBIAL PROPERTIES.

The antimicrobial properties of θ-defensins were first analyzed *in vitro* using bacteria and fungi as test organisms

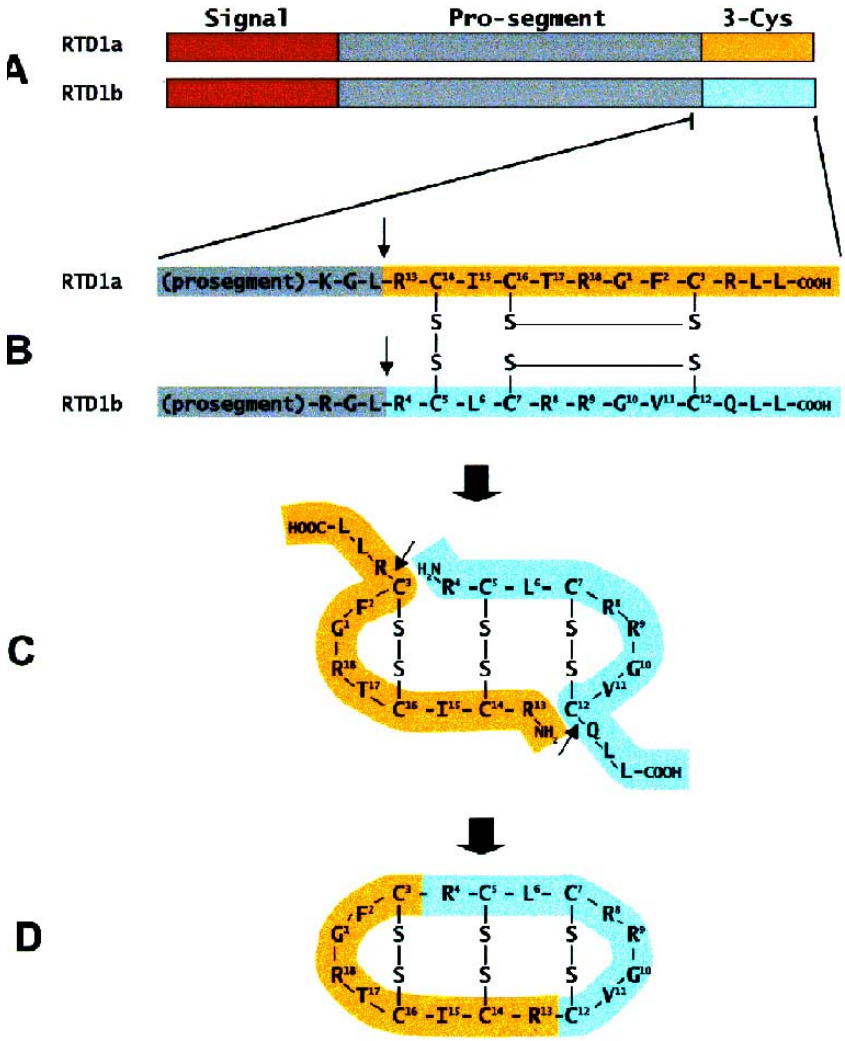

Fig. (5). Trimming and splicing of pro-θ-defensins. The scheme proposed shows (A) diagram of the RTD1a and RTD1b prepro-θ-defensins; the signal peptides are removed leaving the pro-θ-defensins; (B) shows the expanded carboxyl terminal region of each pro-θ-defensin, the putative disulfide that links the two chains, and two intrachain disulfides. Note that in this scheme no additional disulfide rearrangement is necessary. The small arrows are cleavage sites between the pro-segments and the carboxy-terminal dodecapeptides; (C) peptide ligation/cyclization requires concomitant removal of tripeptides from both dodecapeptides (small arrows). It is proposed that these cleavage events are linked to the formation of the two new peptide bonds; (D) mature RTD-1.

[11]. These studies demonstrated that RTD-1 is microbicidal for gram-positive and gram-negative bacteria and fungi (> 99.9% killing) at peptide concentrations in the 1-5 μg/ml range. Moreover, unlike many α- and β-defensins, bactericidal activity was relatively unaffected by addition of physiologic sodium chloride in the incubation mixture. Salt-insensitivity was lost if the peptide backbone was opened (i.e., de-cyclized) at one of the hairpin turns; bactericidal activity of this acyclic RTD-1 analog was completely inhibited by 125 mM NaCl [11]. Further, the antibacterial activity of native RTD-1 was three to four-fold greater than that of the acyclic congener under low salt conditions. RTD-2 and -3 behaved similarly with regard to their antibacterial activities relative to the corresponding acyclic analogs [16].

RTD 1-3 possess similar antimicrobial spectra and activities, despite the fact that RTD-2 (+6) is 50% more cationic than RTD-3 (+4) [16]. Interestingly, RTD-2 was ca. 3-fold less active against *E. coli* than either RTD-1 or RTD-3, despite its greater positive charge. Binding studies were performed to determine whether RTD-2 bound to *E. coli* cells less efficiently than RTD-1 or RTD-3, but no differences were observed [16]. These data suggested that *post-binding* interactions between the different θ-defensins and *E. coli* differentiate the peptides' bactericidal efficacy.

Cole *et al.* recently reported that RTD-1 protected peripheral blood mononuclear cells from infection by both T- and M-tropic strains of HIV-1 *in vitro* [17]. The effect was not the result of virucidal inactivation by the peptide. More recent studies by Munk *et al.*, who analyzed the interaction of RTD-1 analogs, but not RTD-1 *per se*, demonstrate that certain of the analogs exert their anti-HIV-1 effect by inhibiting cellular uptake of the virus. Moreover, these studies demonstrated that the protective effective was highly sequence-specific, as single residue replacements in

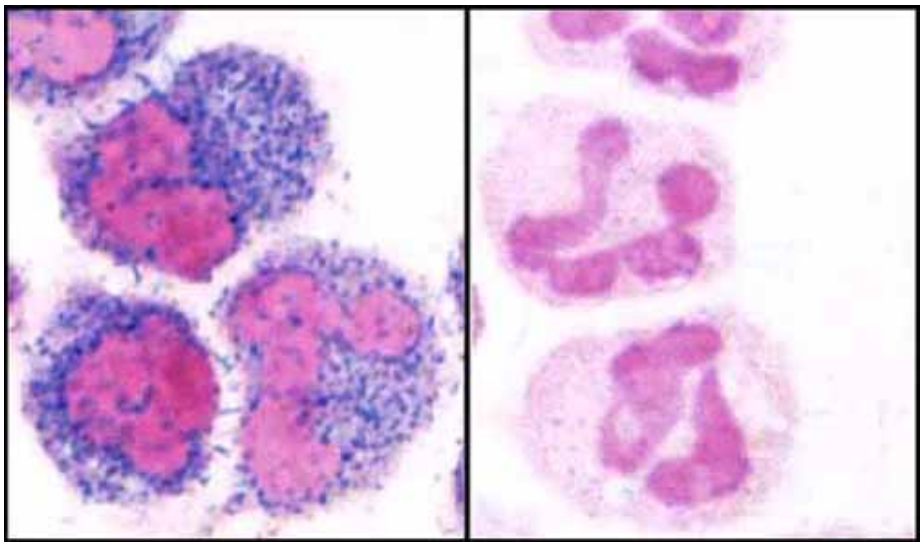

Fig. (6). Cytoplasmic granular localization of RTD-1. Peripheral blood neutrophils were immunostained with anti-RTD-1 IgG (left panel) or pre-absorbed antiserum (right panel) and developed with glucose oxidase/nitroblue tetrazolium, and counterstained with nuclear fast red [11]. The granular staining pattern is identical to that observed when the cells are stained for α-defensins [10].

the θ-defensin peptide backbone dramatically reduced anti-viral potency. Wang *et al.* recently reported that RTD-1 analogs are lectins, and that their antiretroviral activity is due to their ability to bind to CD4 and gp120, molecules that mediate critical interactions in the binding and uptake of HIV by host cells. Thus, theta θ-defensins represent an interesting structural template for the design of topical and/or systemic anti-retrovirals (fully detailed in accompanying paper by Lehrer and Cole [19]).

HUMAN θ-DEFENSINS.

Given that monkeys and humans are ca. 99% identical at the nucleotide level, we predicted that isolation and cloning of human θ-defensins would be a simple matter. However, despite the application of numerous biochemical and genetic strategies, we failed to isolate θ-defensin peptide from human tissues, nor did we identify a pro-θ-defensin ORF in the human genome. However, we did identify a human α-defensin pseudogene, originally reported by Palfree *et al.*, that was an obvious ortholog of RTD1a/1b in which the ORF was interrupted by a stop codon in the signal peptide [20]. We have subsequently characterized two similar human θ-defensin pseudogenes that are transcribed, but which cannot be translated due to the stop codon in the second exon (Fig. **4**). In related experiments we attempted to study the post-translational processing of pro-θ-defensins in HL-60 cells, a human myeloid cell line that properly synthesizes and processes α-defensins. Despite successful transfection of these cells with pro-θ-defensin cDNA, neither the θ-defensin precursor nor the mature peptide could be detected in stable or transient transfectants. These results suggest that one or more elements of the θ-defensin processing were discarded in the evolutionary interval between macaques and humans.

Lehrer and colleagues have investigated the antimicrobial properties of a synthetic version of a resurrected human θ-defensin, termed *retrocyclin*, the sequence of which is composed of a homodimer of nonapeptides derived from the C-terminus of a human θ-defensin pseudogene [Fig. **4**; 17]. As discussed above, retrocyclin and analogs thereof, have been evaluated for their anti-retroviral and lectin-like properties.

CLUES ABOUT ANTIMICROBIAL MECHANISM FROM STRUCTURE

The three dimensional structure of RTD-1, reported by Trabi *et al.* [21] disclosed a conformation composed of two β-strands connected by a pair of hairpin turns, consistent with that proposed in earlier models [11]. The closed loop structure, the presence and distribution of five arginines, and the absence of negative charges confers a topology different from those of α and β-defensins. First, monomeric RTD-1 lacks amphiphilicity [21], whereas α- and β-defensin monomers and/or dimers are amphiphilic, a feature that appears to underlie the ability of these molecules to interact with and disrupt microbial target membranes [22-25]. Interestingly, Huang and colleagues recently showed that RTD-1, like other antimicrobial peptides, bound to lipid bilayers in two different orientations [26]. A unique feature of the RTD-1 binding was the weak membrane thinning observed, an effect that was only observed under conditions where the backbone ring was oriented parallel to the bilayer plane, termed the S-state [26]. The transition of RTD-1 to S-state binding was dependent on a high percentage of hydration of the system, and was irreversible. Trabi *et al.* noted that, in solution, RTD-1 is rather flexible around its central region. Possibly this allows the peptide to bind to polar head groups in a manner that is less disruptive than the more rigid, three dimensionally-constrained backbones of α- and β-defensins. We speculate that the lack of bilayer thinning observed for RTD-1 [26] may underlie the very low cytotoxicity of RTD-1 for human blood mononuclear cells observed *in vitro* [17]. Consistent with this finding, RTD-1

has very low hemolytic activity and low cytotoxicity against fibroblasts (Tran and Selsted, unpublished data).

Certain α-defensins [25] and β-defensins [27, 28] exist as dimeric or higher ordered structures. Interestingly, Trabi *et al*. noted that the NMR data used to define RTD-1 solution structures identified several amide protons of amino acids that might mediate intermolecular interactions [21]. As efforts continue to dissect the molecular basis of the antimicrobial effects of θ-defensins, it will be important to ascertain whether quaternary structural features play a role. Thus, results of crystallographic analyses of θ-defensins may be particularly useful.

BIOSYNTHESIS OF θ-DEFENSINS

There is much interest in identifying the post-translational machinery and processing steps that mediate the cyclization of θ-defensins and the other cyclic peptides discussed in this issue. It is likely that a number of biosynthetic schemes have evolved for this purpose, and that the different pathways that have evolved will be determined by structural features of the precursors that are ultimately cyclized. A case in point is TrbC pilin, a major component of the conjugative bacterial pilus [29]. TrbC, a cyclic 78-mer, is excised and cyclized from a 145-residue precursor. Following removal of a 36 amino acid signal peptide and a 27 residue C-terminal segment, it is proposed that the resulting hydrophobic 82-mer is embedded in cytoplasmic membrane with the amino- and carboxyl-termini in proximity. Cyclization of TrbC is dependent on TraF, a protease that has active-site similarity with signal peptidases. TraF is essential for cleavage of a C-terminal TrbC tetrapeptide, the removal of which appears to be linked to the formation of a peptide bond between the new C-terminal glycine and N-terminal serine. The cyclized molecule then appears to assemble into a pilus structure on the bacterial cell surface. The discovery and characterization of this system is discussed in detail by Kalkum *et al.* in this edition [30].

It is unclear as to whether the example of TrbC cited above might provide hints into the processing pathway of θ-defensins. However, there are some interesting similarities between the maturation of the pilus peptide and RTD 1-3. For example, in both cases the precursors are prepropeptides that are processed at both the amino and carboxyl ends, and the cyclic product is targeted for a new cellular destination. In the case of TrbC, it is a structural element on the cell surface; rhesus θ-defensins are sorted to cytoplasmic granules (Fig. **6**). Most interestingly, however, is the fact that the precursors of both peptides have conserved (putative) cyclization motifs in the C-terminal tetrapeptide (TrbC) or tripeptide (θ-defensins). A linked cleavage-ligation reaction could be operative in both cases, however the molecular mechanism is likely to differ due to the fact that the amino acids involved in the newly formed peptide bonds in θ-defensins are Arg and Cys, rather than Ser and Gly. Unlike the highly hydrophobic TrbC peptide, which possesses two putative transmembrane helices, pro-θ-defensins are quite polar, making it very unlikely that they are membrane bound during the processing events. Moreover, two new peptide bonds must be formed. What orients the constituent chains? One purely speculative scheme would have the unpaired cysteine in each pro-θ-defensin forming a disulfide bond to assemble a dimeric precursor, producing an assembly that is recognized by a protease/ligase complex that carries out the final step(s) that generate the mature cyclic peptide (Fig. **5**).

What evolutionary factors selected for the advent of circularized polypeptides? Trabi and Craik have proposed that conformational stability and resistance to exopeptidases may be the driving forces [31]. In the context of innate immunity and inflammation, it is well-known that proteolytic action is required for the appropriate processing and activation of many antimicrobial peptide precursors, including those of defensins [7, 32-35], and the inflammatory milieu is rich in proteases. Thus, mature antimicrobial peptides have probably evolved to be resistant to digestion in this microenvironment, and in theory, cyclization of the peptide backbone confers absolute immunity of a molecule to exopeptidases. Alternatively, backbone cyclization might be essential for molecular recognition. In this regard, the study by Cole *et al*. is intriguing, as this study demonstrated a dramatic loss of RTD-1 mediated anti-HIV activity associated with θ-defensin de-cyclization [17]. As noted above, de-cyclization of RTD-1 dramatically reduced its bactericidal effect *in vitro*, and eliminated its antibacterial activity in physiologic salt [11].

The discovery that cyclic peptides are produced in animals, as well as in prokaryotes and plants, raises interesting questions regarding the evolutionary need for such molecules. And why did θ-defensins disappear before the appearance of hominids? Are there evolutionary *disadvantages* associated with the production of cyclic polypeptides? Is there a role for such molecules as therapeutic agents? Whatever the answer to these questions, it is clear that there is some very interesting biochemistry and enzymology taking place in the biological systems that produce these cyclic molecules.

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