THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 5, Issue of February 1, pp. 3079–3084, 2002

Printed in U.S.A.

Homodimeric θ-Defensins from *Rhesus macaque* Leukocytes

ISOLATION, SYNTHESIS, ANTIMICROBIAL ACTIVITIES, AND BACTERIAL BINDING PROPERTIES OF THE CYCLIC PEPTIDES*

Received for publication, September 21, 2001
Published, JBC Papers in Press, October 23, 2001, DOI 10.1074/jbc.M109117200

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Rhesus θ-defensin 1 (RTD-1) is a unique tridisulfide, cyclic antimicrobial peptide formed by the ligation of two 9-residue sequences derived from heterodimeric splicing of similar 76-amino acid, α-defensin-related precursors, termed RTD1a and RTD1b (Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., Ouellette, A. J., and Selsted, M. E. (1999) *Science* 286, 498–502). The structures of RTD-2 and RTD-3 were predicted to exist if homodimeric splicing of the RTD1a and RTD1b occurs *in vivo*. Western blotting disclosed the presence of putative θ-defensins, distinct from RTD-1, in leukocyte extracts. Two new θ-defensins, RTD-2 and RTD-3, were purified by reverse-phase high performance liquid chromatography and characterized by amino acid analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, and comparison to the synthetic standards. RTD-2 and RTD-3 are the predicted homodimeric splicing products of RTD1b and RTD1a, respectively. The cellular abundances of RTD-1, -2, and -3 were 29:1:2, indicating that there is a preference for the heterodimeric ligation that generates RTD-1. RTD-1, -2, and -3 had similar antimicrobial activities against *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans*, whereas the activity of RTD-2 against *Escherichia coli* was 2–3-fold less than those of RTD-1 and RTD-3. Equal amounts of each θ-defensin bound to *E. coli* cells, indicating that the differences in antibacterial activities are the result of post-binding processes.

Antimicrobial peptides are essential components of the innate immune system (1–4). They play a significant role at the epithelial defense barrier (5–9) and as the antibacterial arsenals in neutrophils and macrophages (10–13). In mammals, defensins and cathelicidins are the two major antimicrobial peptide families (9, 11, 14). Cathelicidins are heterogeneous peptides that share homology in the proregion with cathelin (14). α- and β-defensins are highly conserved tridisulfide peptides from two genetically distinct families (15, 16). In humans, there are four neutrophilic α-defensins, HNP-1–4 (13); two enteric α-defensins, HD-5 and HD-6 (17); and four epithelial β-defensins, hβD-1–4 (18–22). Although hβD-1 is constitutively expressed in epithelia, the expression of hβD-2, -3, and -4 is inducible by inflammatory cytokines (23) or bacterial infection (18, 21).

The characterization of the host defense components of *Rhesus macaque* granulocytes disclosed two distinct subfamilies of α-defensins (24) and a new tridisulfide peptide termed rhesus θ-defensin 1 (RTD-1)[1] (25). RTD-1 is a macrocyclic 18-amino acid antimicrobial peptide formed by the ligation of two 9-residue sequences derived from similar 76-amino acid, α-defensin-related precursors, termed RTD1a and RTD1b (25). RTD-1 shares some structural similarities with the pig neutrophil protegrins and the horseshoe crab tachyplesins (25–27). The cyclic structure of RTD-1 is an important determinant for microbicidal potency and resistance to the inhibitory effect of physiologic sodium chloride. The antimicrobial potency of synthetic acyclic RTD-1 was 3-fold lower than that of the native peptide under low salt conditions, and the acyclic peptide was completely inhibited by physiologic NaCl (25). Those studies suggested that the cyclic conformation of RTD-1 confers salt-insensitive microbicidal activity that may be critical for antimicrobial function in the extracellular milieu.

The isolation of RTD-1 revealed the existence of a novel post-translational pathway for the production of head-to-tail cyclized peptides in primates. Based on the heterodimeric splicing model that produces RTD-1 from RTD1a- and RTD1b-derived nonapeptides (25), we hypothesized that two additional θ-defensins, termed RTD-2 and RTD-3, would be produced by the homodimeric splicing of RTD1b and RTD1a, respectively. Here we report the isolation of RTD-2 and RTD-3 from circulating leukocytes, as well as the synthesis and antimicrobial and bacterial binding properties of the three rhesus θ-defensins.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Disulfide Formation, and Cyclization—Peptide synthesis was performed essentially as described for RTD-1 (25). Peptide sequences corresponding to open-chain versions of RTD-2 and -3 (see Fig. 1) were assembled at 0.2 mmol scale on Fmoc (9-fluorenylmethoxycarbonyl)-Arg(2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl) polyethylene glycol-polystyrene resin using a Milligen 9050 automated synthesizer. Arg, Cys, and Thr side chains were protected with 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl, triphenylmethyl, and *tert*-butyl groups, respectively. All amino acids except cysteine were coupled with *O*-(7-azabenzotriazol-1-yl)-1,13,3-tetramethyluronium hexafluorophosphate/*N*,*N*-diisopropylethylamine activation. Cysteine residues were coupled as the pre-formed pentafluorophenyl ester derivative. RTD-2 was assembled with double coupling at every cycle. RTD-3 was assembled with double coupling of Thr and Ile residues. Following

* This work was supported by National Institutes of Health Grant AI22931 and a grant from Large Scale Biology, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RTD, rhesus θ-defensin; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectroscopy; AU, acid-urea; HOAc, acetic acid; PIPES, 1,4-piperazinebis(ethanesulfonic acid); TTBS, Tris-buffered saline plus Tween 20 .

chain assembly of RTD-2, the peptide resin was cleaved and deprotected by incubation in 20 ml of reagent R (trifluoroacetic acid:thioanisole:1,2-ethanedithiol; 92:5:3; v/v/v (Ref. 28)) for 4 h at 22 °C with agitation. Cleavage and deprotection of RTD-3 were performed similarly but using reagent K (trifluoroacetic acid:phenol:water:thioanisole:1,2-ethanedithiol; 82.5:5:5:5:2.5; v/w/v/v/v (Ref. 28)). Crude synthetic products were obtained by filtration and extraction with 30% acetic acid/dichloromethane as described for the synthesis of RTD-1 (25).

Linear synthetic RTD-2 and -3 were purified by preparative C_{18} RP-HPLC on a 25 × 100-mm DeltaPak C_{18} cartridge (Waters, MA) developed with a 0.25%/min gradient of water-acetonitrile containing 0.1% trifluoroacetic acid. Aliquots from eluant fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), and those containing reduced/linear peptides were pooled and concentrated 10-fold by centrifugal evaporation. The peptide solutions were diluted to 100–200 μg/ml in 17.4 mM ammonium acetate, pH 8.0, and stirred vigorously in an open container for 18 h at 22 °C. Peptide folding and oxidation were monitored by C_{18} RP-HPLC and MALDI-TOF MS. The acyclic versions of RTD-2 and -3 were then purified by preparative C_{18} RP-HPLC as described above. Purity was confirmed by analytical C_{18} RP-HPLC and acid-urea PAGE on 12.5% polyacrylamide gels (29). For MALDI-TOF MS, peptide solutions were mixed with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50/50 water-acetonitrile containing 0.1% trifluoroacetic acid and analyzed on a Voyager DE-RP mass spectrometer (PerSeptive Biosystems) (25).

Acyclic RTD-2 (10 mg) and RTD-3 (5 mg) were lyophilized, first from 25 mM hydrochloric acid (three times), then from distilled water (twice). Peptide cyclization was carried out by dissolving the lyophilized peptides at 200–300 μg/ml in 0.1% diisopropylethylamine/dimethyl sulfoxide (v/v) containing 60 molar eq of 1-ethyl-3-[3-dimethylaminopropyl-]carbodiimide and 20 eq of 1-hydroxybenzotriazole. The solutions were sealed under nitrogen and stirred for 18 h at 22 °C. The extent of peptide cyclization was determined by C_{18} RP-HPLC and MALDI-TOF MS. Cyclic peptides were purified to homogeneity by C_{18} RP-HPLC with water-acetonitrile gradients (0.25%/min) containing 0.1% trifluoroacetic acid and characterized by analytical C_{18} RP-HPLC, AU-PAGE, MALDI-TOF MS, and amino acid analysis (25).

Antibody Production—Rabbit anti-RTD-3 antibody was produced as described previously for the preparation of anti-RTD-1 antibody (25). Briefly, acyclic RTD-3 (3.5 mg) was conjugated to ovalbumin (3.5 mg) with 0.1% glutaraldehyde in 7 ml of 100 mM sodium phosphate, pH 7.4, and stirred for 18 h at 22 °C. The reaction was quenched with 300 mM glycine, and the peptide/ovalbumin conjugate was dialyzed exhaustively against water. Two New Zealand White rabbits were repeatedly immunized using standard procedures until the anti-RTD-3 antiserum titer was 1:10,000, as determined by enzyme-linked immunosorbent assay. IgG-enriched preparations were obtained by chromatography on a DEAE Econo-Pac column according to the manufacturer's protocol (Bio-Rad).

Western Blot Analysis—Five percent acetic acid (HOAc) extracts of 1×10^7 leukocytes were resolved on a 12.5% acid-urea polyacrylamide gel and electroblotted to a 0.22-μm nitrocellulose membrane with an LKB Novablot apparatus (Amersham Biosciences, Inc.) using the semi-dry transfer method (30). Replica blots were blocked with 5% nonfat dried milk in TTBS (100 mM Tris buffer, pH 7.5, containing 0.9% sodium chloride and 0.1% Tween 20) for 1 h at 22 °C with agitation and incubated with a 1:150 dilution (in TTBS) of rabbit anti-RTD-1 (25), anti-RTD-3, or normal rabbit IgG for 1 h. Blots were washed with TTBS (five 10-min washes), and developed with the ABC-Elite kit (Vector Laboratories) as follows: 30-min incubation in biotinylated goat anti-rabbit IgG diluted 1:2,800 in TTBS, 5-min washes (three times), and 30-min incubation in a 1:10 dilution of the avidin-horseradish peroxidase reagent. Immunopositive bands were visualized with Supersignal chemiluminescent substrate (Pierce) on Hyperfilm (Amersham Biosciences, Inc.).

Purification of Natural RTD-1–3—Peripheral blood leukocytes (>90% PMN) were obtained from four rhesus monkeys. Approximately $1–1.8 \times 10^8$ leukocytes/animal were extracted with 5% HOAc as described previously (24, 25). Pooled acid extracts of $1–10 \times 10^7$ cell eq were fractionated on a 4.6 × 250-mm Vydac C_{18} column using water-acetonitrile gradients (0–30% acetonitrile in 70 min) containing 0.1% trifluoroacetic acid or 0.1% phosphoric acid (H_3PO_4). Eluant fractions were analyzed by MALDI-TOF MS for peptides with molecular masses of RTD-1–3. Three θ-defensins were purified to homogeneity by successive rounds of RP-HPLC using the same solvent gradient but alternating between 0.1% trifluoroacetic acid and 0.1% H_3PO_4 as the ion pairing reagent.

Purified RTD-1–3 were characterized by MALDI-TOF MS, amino acid analysis, and AU-PAGE. Cysteine content was determined by comparing the masses of the native peptides with those obtained following reduction of disulfides with 1,4-dithiothreitol and alkylation with iodoacetamide (31). The amino acid compositions of RTD-1–3 were determined on 6 N HCl hydrolysates (2 h, 150 °C) as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatives (32). The absence of free N termini in natural RTD-2 and -3 was determined by Edman sequence analysis on 20–50 pmol of each peptide. Natural peptides were compared with synthetic versions by RP-HPLC, AU-PAGE, amino acid analysis, and MALDI-TOF mass spectroscopy.

Antimicrobial Activities of RTD-1–3—The antimicrobial activities of synthetic RTD-1–3 against bacteria (*Staphylococcus aureus* 502a and *Escherichia coli* ML35) and fungi (*Candida albicans* 16820 and *Cryptococcus neoformans* 271A) were determined in an agar diffusion assay as described previously (33). Briefly, 10-μl wells were bored in a 9-cm^2 plate of agarose, buffered with 10 mM PIPES, pH 7.4, containing 5 mM glucose, and seeded with 1×10^6 mid-log phase cells. Five-μl aliquots of each peptide, dissolved in 0.01% HOAc at 10–300 μg/ml, were added to each well. After incubation at 37 °C for 2 h, the seeded agar was overlaid with molten agarose containing 6% trypticase soy broth (for bacteria) or Sabouraud dextrose broth (for fungi). Plates were incubated at 37 °C for 18–24 h, and antimicrobial activity was determined by measuring the diameter of clearing around each well.

The microbicidal activities of each peptide were determined by incubating 2×10^6 colony-forming units/ml with peptides (0.5–12 μg/ml) in 50 μl of low salt diluent, 10 mM PIPES buffer containing 5 mM glucose, pH 7.4, or the same diluent supplemented with 25–150 mM NaCl. After 2 h of incubation at 37 °C, the cell suspensions were diluted 1:50 with 10 mM sodium phosphate buffer, pH 7.4, and exponentially spread with an Autoplate 400 (Spiral Biotech) onto trypticase soy agar (bacteria) or Sabouraud dextrose agar (fungi). After incubation at 37 °C for 18–48 h, colonies were counted and cell survival was expressed as colony-forming units/ml.

Binding of RTD-1–3 to *E. coli* ML35 was evaluated by incubating 2×10^6 log-phase bacteria with increasing peptide concentrations (0.5–8 μg/ml final) in 1 ml of 10 mM PIPES, pH 7.4, containing 5 mM glucose for 2 h at 37 °C. The incubation mixtures were centrifuged at 20,000–25,000 × *g* for 10 min at 22 °C, and supernatant samples were analyzed for RTD content by RP-HPLC. Binding of each RTD to *E. coli* was determined by subtraction of the quantity of supernatant peptide from total added to each tube, and comparing this to control incubations lacking bacteria.

RESULTS

Peptide Synthesis, Disulfide Formation, and Cyclization—RTD-2 and RTD-3 are cyclic analogs of RTD-1 predicted to be produced by homodimeric splicing of nonapeptides from RTD1b and RTD1a, respectively (Fig. 1). Linear RTD-2 and -3 were synthesized and purified by preparative RP-HPLC. Disulfide bond formation proceeded efficiently in room air, giving >90% yield of monomeric, tridisulfide peptide as determined by quantitative RP-HPLC and MALDI-TOF MS. The yields of the subsequent peptide cyclization steps were 92% for RTD-2 and 64% for RTD-3. The cyclic peptides were purified by C_{18} RP-HPLC and characterized by AU-PAGE, amino acid analysis, and MALDI-TOF MS. RTD-2 (9.2 mg) and RTD-3 (3.2 mg) preparations were more than 99% pure, and were indistinguishable from the natural peptides (see below).

Isolation of Natural RTD-1–3—Synthetic RTD-1, -2, and -3 had unique R_F values on acid-urea PAGE because of their differing arginine contents (Fig. 2). Acid extracts of rhesus macaque leukocytes contained a band that co-migrated with synthetic RTD-1 on AU-PAGE and Western blots. RTD-1 and two additional immunopositive bands that co-migrated with RTD-2 and RTD-3 synthetic standards were detected in leukocyte extracts with anti-RTD-1 and anti-RTD-3 antibodies (Fig. 2). These data strongly suggested the presence of RTD-2 and RTD-3 in leukocyte extracts.

RTD-1–3 were isolated from leukocyte extracts by RP-HPLC (Fig. 3). Peptides with masses of RTD-1, -2, and -3 were detected in three peaks following the initial chromatographic step (Fig. 3*A*), and the RP-HPLC elution times precisely matched

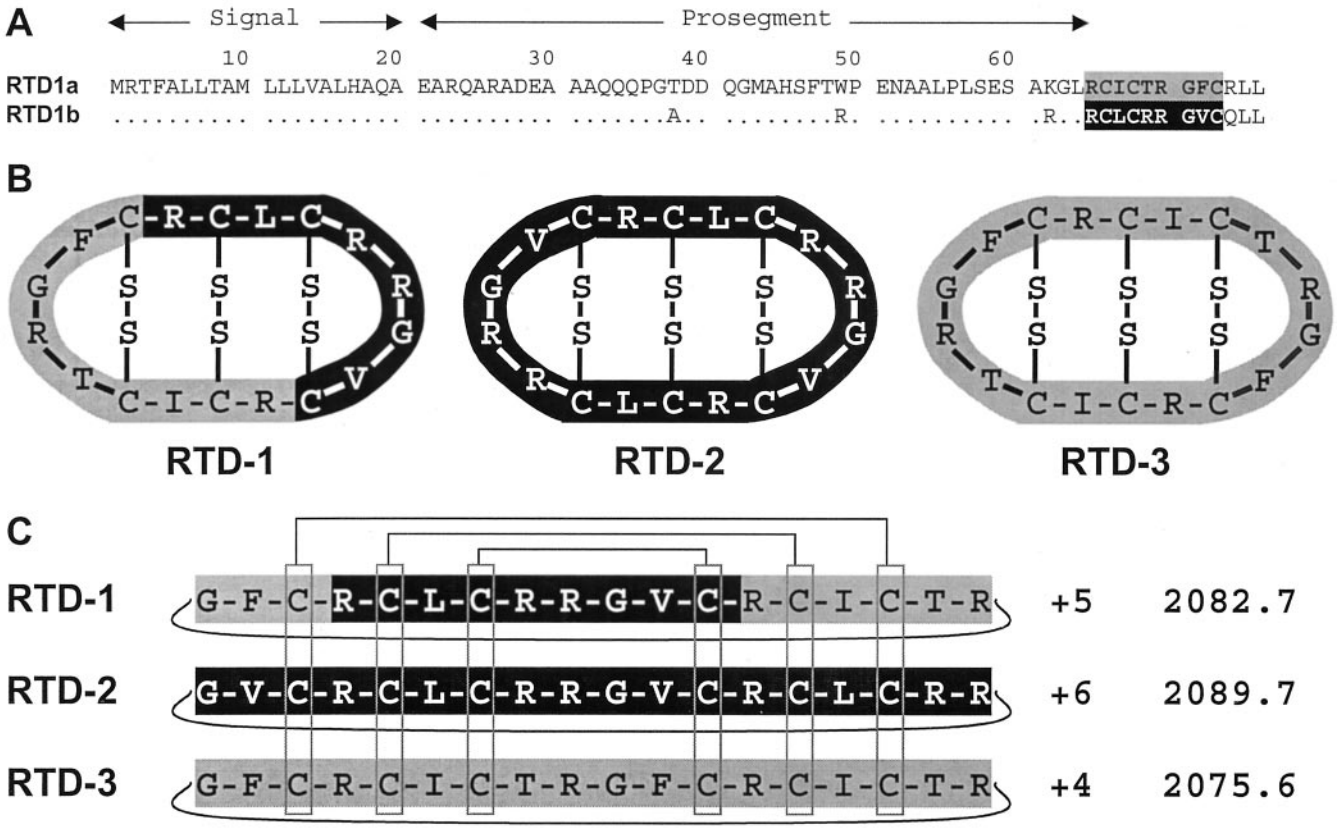

FIG. 1. **The structures of rhesus θ-defensins and precursors.** *A*, the cDNA-deduced amino acid sequences of θ-defensin precursors, RTD1a and RTD1b (25). The RTD1a- and RTD1b-derived nonapeptides that contribute to the final products are highlighted in *gray* and *black*, respectively. *B*, the cyclic structures of heterodimeric (RTD-1) and homodimeric (RTD-2 and RTD-3) θ-defensins are shown employing the scheme of *A*. *C*, alignment of RTD-1–3 amino acid sequences. The calculated peptide net charges and the theoretical masses are shown.

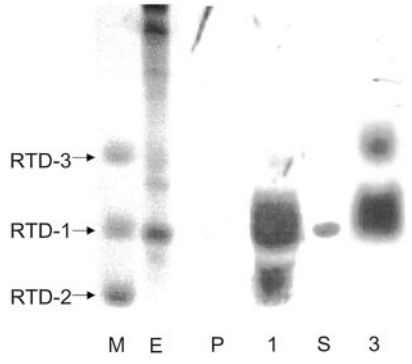

FIG. 2. **Detection of rhesus θ-defensins in acid extracts of rhesus leukocytes by AU-PAGE and Western blotting.** A mixture (*lane M*) of synthetic RTD-1–3, 0.5 μg each, and acid extract (*lane E*) of 2×10^6 rhesus leukocytes were resolved on a 12.5% acid-urea polyacrylamide gel and stained with formalin-Coomassie Blue. Lanes containing acid extracts of 1×10^7 leukocytes (*lanes P*, *1*, and *3*) and 0.5 μg of synthetic RTD-1 (*lane S*) were transferred to nitrocellulose membrane for Western blotting (see "Experimental Procedures"). Replica blots were incubated with rabbit pre-immune (*lane P*), anti-RTD-1 (*lane 1*), or anti-RTD-3 (*lane 3*) antibodies.

those of the respective synthetic peptides. Each θ-defensin was purified to homogeneity (Fig. 3*B*), and their identities were confirmed as described below.

Characterization of RTD-2 and -3—Automated Edman degradation of 20–50 pmol of purified RTD-2 and RTD-3 yielded no amino acid signal, consistent with the θ-defensin cyclic structure. The molecular masses of natural RTD-2 and RTD-3, determined by MALDI-TOF MS, matched the calculated values of the predicted sequences (Fig. 1). The cysteine content of purified RTD-1–3 was determined by comparing the molecular masses of native peptides with those that had been reduced and alkylated. Carboxamidomethylated RTD-1, -2, and -3 had molecular masses of 2430.5 atomic mass units (2430.7 = theoretical), 2436.9 atomic mass units (2437.7 = theoretical), and 2424.5 atomic mass units (2423.6 = theoretical), respectively, consistent with the complete alkylation of 6 cysteine residues in each θ-defensin (Table I).

The compositions of natural RTD-2 and -3, determined by amino acid analysis of peptide hydrolysates (Table I), were consistent with those of the corresponding structures shown in Fig. 1 (*B* and *C*), and that of purified RTD-1, which was as reported previously (25). In a previous study, synthetic RTD-1 was biochemically and functionally equivalent to the natural peptide (25). Synthetic RTD-2 and -3 were also indistinguishable from the natural isolates by amino acid analysis, MALDI-TOF MS, AU-PAGE, and analytical RP-HPLC (Fig. 4).

The amount of each θ-defensin isolated from acid extracts of rhesus leukocytes was determined by quantitative amino acid analysis. The cellular abundance of θ-defensins in extracts of 5.8×10^8 cell eq was 107 μg (51.4 nmol) of RTD-1, 3.8 μg (1.8 nmol) of RTD-2, and 8.8 μg (4.2 nmol) of RTD-3, giving relative cellular abundances of 29:1:2 (RTD-1:RTD-2:RTD-3). These data indicate that 10-fold more heterodimeric RTD-1 is present in cells than the homodimeric homologs.

Antimicrobial Activities of RTD-1–3—An agar diffusion assay was utilized to assess the combined microbicidal and microbistatic activities of RTD-1–3 against *Staphylococcus aureus* 502a, *Escherichia coli* ML35, and yeast forms of *Candida albicans* 16820 and *Cryptococcus neoformans* 271A. The antimicrobial activities of the three θ-defensins were equivalent against *S. aureus*, *C. albicans*, and *C. neoformans*. RTD-2 was 2–3-fold less active than RTD-1 and RTD-3 against *E. coli* (Fig. 5). The

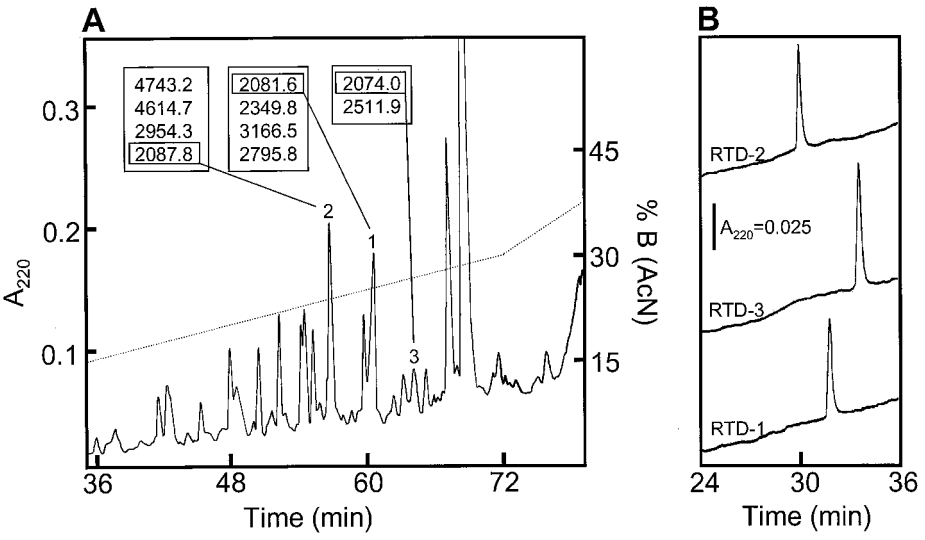

FIG. 3. **Purification of RTD-1–3.** *A*, acid extracts of 4×10^7 leukocytes were chromatographed on a C_{18} reverse-phase column using a 0.5%/min water-acetonitrile gradient containing 0.1% trifluoroacetic acid. *Numbered peaks* contained peptides with molecular masses shown, including masses consistent with RTD-1, -2, and -3. *B*, purified RTD-1–3 were chromatographed on a C_{18} column using a 1%/min water-acetonitrile gradient containing 0.1% trifluoroacetic acid.

TABLE I
Amino acid compositions of rhesus θ-defensins

	RTD-1	RTD-2	RTD-3
Amino acid			
Arg	4.84 (5)	5.63 (6)	3.78 (4)
Cys	6.00 (6)	6.00 (6)	6.00 (6)
Gly	2.03 (2)	2.20 (2)	2.19 (2)
Ile	0.96 (1)	0 (0)	2.19 (2)
Leu	1.01 (1)	2.00 (2)	0 (0)
Phe	1.05 (1)	0 (0)	2.00 (2)
Thr	1.00 (1)	0 (0)	1.94 (2)
Val	0.98 (1)	1.92 (2)	0 (0)
Molecular mass			
Cyclized, oxidized			
Calculated	2082.7[a]	2089.7[a]	2075.6[a]
Experimental	2083.0[b]	2087.9[b]	2076.0[b]
Linear, reduced			
Calculated	2106.7[c]	2113.7[c]	2099.6[c]
Difference	24[d]	24[d]	24[d]

[a] Calculated based on cyclic structures shown in Fig. 1 (*B* and *C*).
[b] Determined by MALDI-TOF mass spectroscopy.
[c] Calculated based on amino acid compositions of linear peptides.
[d] Differences between *a* and *c*, accounted for by loss of 18 atomic mass units resulting from cyclization and loss of 6 atomic mass units because of disulfide formation.

microbicidal potencies of RTD-1, -2, and -3 were determined in liquid-phase assays (see "Experimental Procedures"). The bactericidal and fungicidal activities of the three peptides were similar. However, approximately twice as much peptide was required to kill fungal suspensions as was needed to kill the same number of bacterial cells (Fig. 6). RTD-2-mediated killing of *E. coli* showed a steep dose dependence similar to that of RTD-1 and -3, but approximately twice as much RTD-2 was required to achieve the microbicidal activities of RTD-1 and -3. However, at concentrations of 2 μg/ml or higher, all three θ-defensins reduced the viability of *E. coli* ML35 by at least 99.9%.

Unlike most α- and β-defensins, the microbicidal activity of RTD-1 is relatively unaffected by physiologic NaCl (25). The microbicidal activities of 10 μg/ml of RTD-2 and RTD-3 were determined using *S. aureus* 502a and *E. coli* ML35 as test organisms in the presence of 0–150 mM NaCl. As with RTD-1, the potent microbicidal activity of RTD-2 and RTD-3 was unaffected by NaCl at all salt concentrations tested (data not shown). The activities of θ-defensins were also compared with those of other potent antibacterial peptides. Microbicidal assays were performed in parallel with indolicidin (a linear, tryptophan-rich, bovine cathelicidin; Refs. 34 and 35) and mouse enteric α-defensins (cryptdins) 3 and 4. On a molar basis, RTD-1–3 were each 2–4 times as potent as indolicidin against *S. aureus* 502a, and each θ-defensin was equipotent to the cryptdins against *S. aureus* (data not shown). A (29:1:2) mixture of synthetic RTD-1, -2, and -3 was prepared and used in microbicidal assays against the four test organisms. The peptide mixture exhibited microbicidal potencies that were identical to that of RTD-1 (data not shown), indicating that synergistic microbicidal interactions do not occur under these assay conditions.

Experiments were performed to determine whether the difference activities of RTD-1–3 against *E. coli* ML35 are the result of differential binding of the peptides to the bacterium. As shown in Fig. 7, the binding of the three θ-defensins to *E. coli* cells was essentially identical. For all three θ-defensins, maximal peptide binding by 2×10^6 bacteria was ~2 μg. The corresponding peptide concentrations (0–2 μg/ml) are associated with dose-dependent killing of bacterial and fungal targets (Fig. 6). However, despite the equal binding by RTD-1–3 to *E. coli* cells, the colicidal activity of RTD-2 is substantially lower than that of RTD-1 and -3 (Fig. 6). Thus, other peptide-cell interactions influence the relative microbicidal potencies of the three θ-defensins.

DISCUSSION

Macrocyclic peptides composed entirely of L-amino acids are relatively rare biomolecules, nearly all known examples of which have been isolated from plants. More than 30 macrocyclic peptides, collectively termed *cyclotides*, have been identified in plants of the Rubiaceae and Violaceae families (36). The mature active peptides typically contain 30 amino acids including 6 cysteines connected in a 1–4, 2–5, 3–6 motif. The structures of two cyclotides (circulin A and cycloviolacin O1), determined by NMR, contain several β-strands constrained by a cysteine knot (36, 37). Peptide folding kinetics of acyclic permutants indicated that the intact cysteine knot is critical for native conformation. Biosynthesis of plant cyclotides involves the post-translational ligation and splicing of the polypeptide precursors (38, 39). The observation that some cyclotides possess antimicrobial activities suggests that these peptides may have a role in plant defense (40, 41).

RTD-1 was the first macrocyclic antimicrobial peptide discovered in animals, disclosing the capacity of animal cells to produce this circular motif (25). The fact that RTD-1 is produced by the heterodimeric ligation of two nonidentical nonapeptides encoded by distinct genes suggested that homodimers might be formed similarly. The isolation of RTD-2 and RTD-3 confirmed this hypothesis. Moreover, the biosyn-

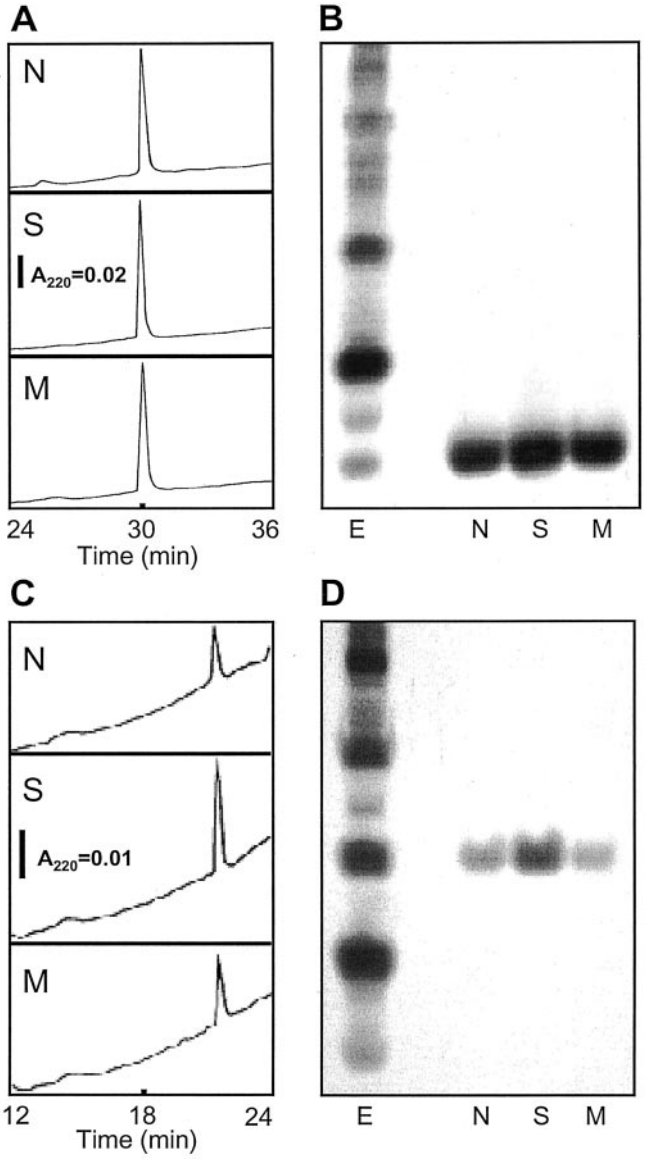

FIG. 4. **Comparison of natural and synthetic RTD-2 and RTD-3.** Natural (*N*) RTD-2 (1.0 μg; *panel A*) and RTD-3 (0.5 μg; *panel C*) and 1.0 μg of each of the corresponding synthetic (*S*) versions were analyzed individually or as 1:1 mixtures (*M*) by C_{18} RP-HPLC. Eluant peaks were collected and analyzed by AU-PAGE as described in Fig. 2 legend (RTD-2, *panel B*; RTD-3, *panel D*). Acid extracts of 2×10^6 rhesus leukocytes (*E*) were electrophoresed in parallel for comparison.

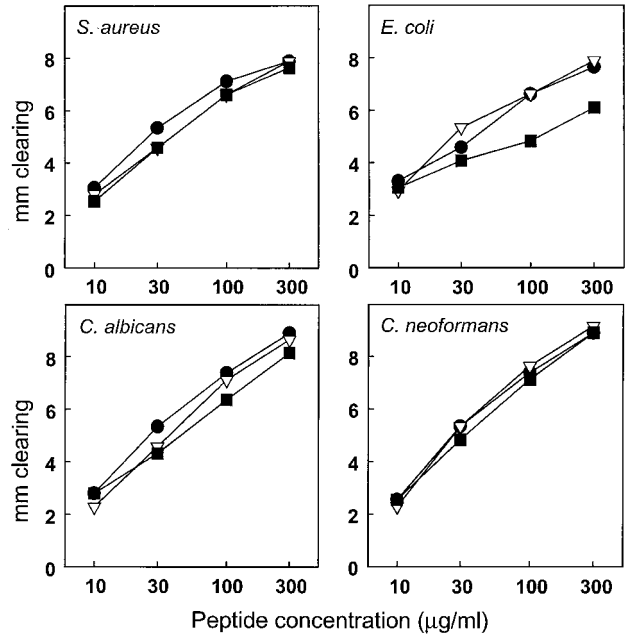

FIG. 5. **Antimicrobial activities of RTD-1–3.** The antimicrobial activities of synthetic RTD-1 (●), RTD-2 (■), and RTD-3 (▽) against *S. aureus* 502a, *E. coli* ML35, *C. albicans* 16820, and *C. neoformans* 271a were assessed in an agar diffusion assay as described under "Experimental Procedures."

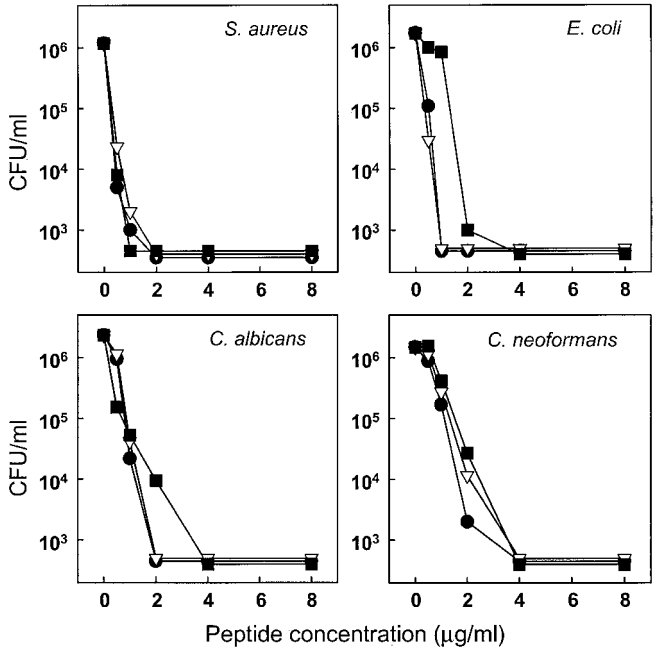

FIG. 6. **Microbicidal activities of RTD-1–3.** Each organism was incubated with increasing concentrations of RTD-1 (●), RTD-2 (■), or RTD-3 (▽) in 10 mM PIPES, pH 7.4, containing 5 mM glucose for 2 h at 37 °C. The limit of detection (1 colony/plate) was equal to 1×10^3 colony-forming units/ml in the incubation mixture.

thesis of three unique θ-defensins suggests that the cellular machinery responsible for peptide cyclization may be involved in post-translational modification of other gene products (25).

The cyclic structures of RTD-1–3 endow the peptides with resistance to exoproteinases, and this may be advantageous in a protease-rich inflammatory milieu. Furthermore, peptide cyclization confers other properties to RTD-1 (25) that are absent in an acyclic analog, because acyclic RTD-1 is substantially less active than the native peptide against *S. aureus* and *E. coli* (25). RTD-1–3, but not the respective acyclic analogs, maintain their staphylocidal and colicidal activities in the presence of physiologic sodium chloride (Ref. 25 and data not shown).

The relative yields of RTD-1, -2, and -3 obtained from leukocyte extracts indicated that RTD-1 is 10-fold more abundant than RTD-2 and -3 combined. This suggests a strong preference for production of RTD-1 by heterodimeric splicing of the RTD-1 precursors, RTD1a and RTD1b. We speculate that distinct elements in the two precursors may direct the assembly of heterodimeric intermediates prior to peptide splicing (25).

Despite differences in cationicity, RTD-1 (+5), RTD-2 (+6), and RTD-3 (+4) possess similar antimicrobial potencies against four organisms tested in this study, and were nearly identical against *S. aureus, C. albicans*, and *C. neoformans*. The most cationic peptide, RTD-2, was slightly less active against *E. coli* than RTD-1 and RTD-3. This was somewhat surprising because increased cationicity typically correlates with greater antimicrobial activities and increased spectrum of

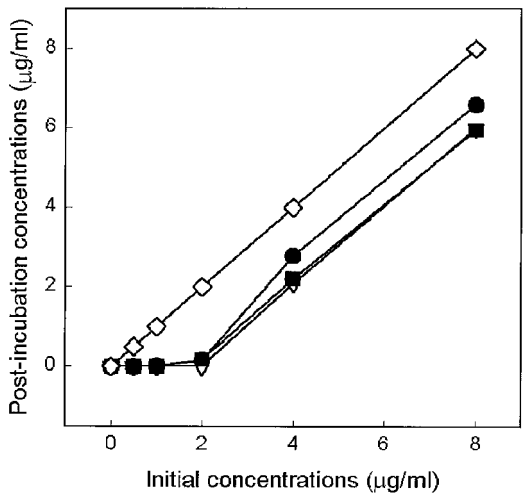

FIG. 7. **Binding of RTD-1–3 to *E. coli* ML35.** Varied concentrations of RTD-1 (●), RTD-2 (■), or RTD-3 (▽) were incubated with 1×10^6 colony-forming units/ml log-phase bacteria in 1 ml of 10 mM PIPES, pH 7.4, containing 5 mM glucose for 2 h at 37 °C. The amount of each peptide in the supernatant was determined by quantitative C_{18} RP-HPLC and compared with the "no-bacteria" control (◇) as described under "Experimental Procedures."

activity (42, 43). However, the activity of RTD-2 against *E. coli* was nearly equivalent to those of RTD-1 and RTD-3 when longer incubation times (4 or 6 h) were used in the agar diffusion assay (data not shown). This suggests that the kinetics of peptide-bacteria interactions differs among the three θ-defensins under these assay conditions.

The binding of peptide to *E. coli* ML35 was equivalent for all three θ-defensins (Fig. 7), indicating that difference in bactericidal activities of RTD-1–3 (Figs. 5 and 6) is the result of subsequent interactions of peptides with bacterial cells. We speculate that the increased electrostatic interaction of the more cationic RTD-2 with components of the *E. coli* cell envelope adversely affects the antibacterial activity. An alternative explanation is that quaternary interactions occur once the peptide is bound to the target cell, and that those interactions are less favored in the case of the highly cationic RTD-2.

The studies presented here demonstrate that RTD-1–3 are broad spectrum microbicides that kill bacterial and fungal targets in a salt-insensitive manner. Under the conditions of the *in vitro* assay system, synergistic antimicrobial activity was not apparent. However, it is possible that the activities of θ-defensins, individually or in combination, may be substantially altered by the microenvironment in which they function *in vivo*.

Addendum—As the current studies were being prepared for publication, we became aware of a report by Leonova *et al.* (44), who described the isolation of RTD-1–3 from rhesus macaque bone marrow, the structures of which are identical to those reported here. Further, the solution structure of synthetic RTD-1 was recently reported by Trabi *et al*. (45), fundamentally confirming the model proposed previously (25).

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