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Protegrins: Structural Requirements for Inactivating Elementary Bodies of *Chlamydia trachomatis*

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We tested 20 protegrins against *Chlamydia trachomatis* serovar L2 (L2/434/Bu). Five of the protegrins had native structures; the others included nonamidated, enantiomeric, and truncated variants and peptides with <2 disulfide bonds. Antichlamydial activity resided principally in residues 5 to 15 of native protegrin PG-1, and optimal activity required both intramolecular disulfide bonds.

Cysteine-rich, endogenous antimicrobial peptides are widely distributed in the phagocytic cells and epithelial tissues of humans and other animals. For example, the neutrophils and small intestinal Paneth cells of humans contain α-defensins, a family of 3.5-kDa peptides with cysteine-stabilized β-sheet structures and broad-spectrum antimicrobial efficacy (2, 9). The female human genital tract produces hBD-1 (1), a β-defensin homologous to the antimicrobial peptides found in bovine (13) and avian (5, 6) leukocytes and various bovine epithelial cells (4, 12). Protegrins, the subject of this study, are unusually potent antimicrobial molecules that were originally isolated from porcine leukocytes (7). They are active against several important sexually transmitted pathogens, including *Neisseria gonorrhoeae* (11), *Chlamydia trachomatis* (15), and human immunodeficiency virus type 1 (14). Like defensins, protegrins possess a cystine-stabilized β-sheet structure; however, they are much smaller than defensins and contain only two intramolecular disulfide bonds. We initiated this study to determine if structurally simpler, protegrin-like molecules would retain antichlamydial activity.

Peptides. Synthetic PG-1 amide, PG-1 acid, enantio-PG-1, PG-2, PG-3, and PG-5 were prepared by SynPep (Dublin, Calif.), and purified as previously described (11, 15). The other protegrin variants used in this study were synthesized by Fmoc chemistry at the Macromolecular Structure Facility of the University of Arizona, which monitored the fidelity of their synthesis by reverse-phase high-performance liquid chromatography (RP-HPLC) and fast atom bombardment mass spectrometry. These peptides were reduced with dithiothreitol in our laboratory, purified by RP-HPLC, and dissolved in 0.1 M Tris buffer, pH 7.7, at a peptide concentration of 0.1 to 0.3 mg/ml. The reduced peptides were oxidized at 23°C for 24 to 48 h in room air to allow formation of their intramolecular disulfide bonds. Dimethylsulfoxide, 10% (vol/vol) final concentration, was added when uni-disulfide protegrin variants were oxidized, to enhance the efficiency of S-S bond formation. All peptides used in these studies were at least 98% pure, as judged by analytical RP-HPLC and acid-urea-polyacrylamide gel electrophoresis. The purified peptides were prepared and stored as 1-mg/ml stock solutions in sterile acidified water (0.01% glacial acetic acid).

Chlamydiae. *C. trachomatis* serovar L2 (L2/434/Bu) was prepared as previously described (8) and used in all experiments. The seed inoculum was prepared by sonicating mouse L929 fibroblasts (ATCC CCL1) infected in tissue culture. The titer of the seed was determined at the time of preparation and adjusted appropriately to produce countable numbers of inclusion-forming units (IFU). All reported experiments were repeated two or three times with duplicate or triplicate coverslips.

TABLE 1. Inactivation of *C. trachomatis* by full-length (18 residues) protegrins

Peptide	Sequence[a]	No. of residues	No. of expt[b]	IC_{50} (μg/ml) (95% C.I.)[c]	Relative potency	Mean % IFU (μg/ml)[d]			
						31.6	10.0	3.16	1.0
PG-1	RGGRLCYCRRRFCVCVGR*	18	15	2.1 (1.76–2.40)	1.00	98.5 (0.48)	88.5 (1.3)	66.0 (2.2)	23.1 (6.7)
PG-3	RGG**G**LCYCRRRFCVCVGR*	18	2	2.0 (1.16–3.43)	1.10	99.2 (0.8)	89.1 (3.1)	64.1 (10.3)	28.7 (14.1)
PG-4	RGGRLCYCR**GWI**C**F**CVGR*	18	2	3.5 (3.07–3.89)	0.62	99.8 (0.17)	84.3 (0.35)	47.1 (2.1)	17.0 (10.1)
PG-5	RGGRLCYCR**P**RFCVCVGR*	18	2	2.2 (1.11–4.33)	0.95	99.0 (0.6)	73.9 (178)	58.0 (9.3)	32.7 (12.4)
PG-1 acid	RGGRLCYCRRRFCVCVGR□	18	2	2.1 (1.99–2.21)	1.00	97.7 (0.2)	86.4 (0.6)	62.8 (0.75)	26.9 (1.8)
Enantio PG-1	RGGRLCYCRRRFCVCVGR* (D)	18	3	1.9 (1.87–1.95)	1.10	98.2 (0.8)	89.4 (0.5)	70.6 (0.35)	23.6 (0.9)

[a] The primary sequences are shown in standard single-letter code. The asterisk (*) signifies the presence of C-terminal amidation, and the square (□) denotes its absence. Residues which differ from the corresponding ones in PG-1 are shown in boldface.

[b] Experiments were set up with triplicate cover slips per concentration. IC_{50} denotes the peptide concentration that caused 50% reduction in IFU, relative to control, and was used to calculate a peptide's relative potency versus PG-1, which was assigned a value of 1.00.

[c] C.I., confidence interval.

[d] SEM values are in parentheses.

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TABLE 2. Inactivation of *C. trachomatis* by truncated protegrins[a]

Peptide	Sequence[b]	No. of residues	No. of expts	IC_{50} (μg/ml) (95% C.I.)	Relative potency	Mean % IFU (μg/ml)[c]			
						100.0	31.6	10.0	3.16
PG-1	RGGRLCYCRRRFCVCVGR*	18	15	2.1 (1.76–2.40)	1.00	100 (0.0)	98.5 (0.48)	88.5 (1.3)	66.0 (2.2)
PG-2	RGGRLCYCRRRFC**I**CV*	16	2	2.6 (2.26–3.05)	0.81	100 (0.0)	100 (0.0)	96.5 (0.25)	55.3 (1.7)
PC-13	RGGRLCYCRRRFCVCV--*	16	3	4.2 (3.46–4.90)	0.50	99.9 (0.07)	97.6 (1.25)	81.8 (3.1)	40.5 (a) (4.0)
PC-45	RGGRLCYCRRRFCVC---*	15	3	4.6 (2.80–7.47)	0.42	97.1 (c) (1.4)	81.6 (c) (3.8)	57.4 (c) (4.1)	46.5 (2.8)
PC-11	----LCYCRRRFCVCVGR*	14	3	4.5 (1.86–10.6)	0.45	99.4 (b) (0.5)	92.1 (a) (2.7)	70.6 (a) (5.0)	41.3 (15.6)
PC-37	-----CYCRRRFCVCVGR*	13	3	17.4 (10.1–29.9)	0.11	96.2 (c) (1.9)	71.0 (c) (0.9)	30.7 (c) (18.3)	25.2 (b) (15.2)
PC-17	----LCYCRRRFCVCV--*	12	3	15.5 (12.0–20.1)	0.13	98.6 (c) (0.7)	73.4 (c) (2.9)	35.6 (c) (6.7)	22.9 (d) (1.9)
PC-73	-----CYCRRRFCVC---*	10	3	47.2 (29.9–74.5)	0.04	66.1 (c) (3.0)	41.4 (c) (7.3)	1.6 (c) (9.2)	−0.5 (c) (8.5)

[a] The organization of the data is as described for Table 1.
[b] Deleted residues are represented by a dash (−). PG-2, a naturally occurring protegrin, differs from PC-13 only by a single isoleucyl residue, which is shown in boldface.
[c] Each peptide's activity at the indicated concentration was compared to that of PG-1 by an unpaired *t* test. (a) signifies $P < 0.05$, (b) signifies $P < 0.01$, and (c) signifies $P < 0.001$.

The numbers of *C. trachomatis* IFU in peptide-treated and control cultures were compared by Student's *t* test for unpaired samples (two-tailed). The 50% inhibitory concentration (IC_{50}) values were calculated by linearly interpolating (log-transformed) peptide concentration versus (linear scale) inhibitory activity data, using the 2 concentrations of peptide that flanked the IC_{50}. The IC_{50} is a ratio, whose standard error depends on the standard error of the mean (SEM) of the activity levels and whose variance can be calculated from the formula for variance of a ratio:

$$\mathrm{Var}\left(\frac{A}{B}\right) = \left(\frac{A}{B}\right)^2 \left(\frac{\mathrm{Var}(A)}{A^2} + \frac{\mathrm{Var}(B)}{B^2} - 2\frac{\mathrm{Cov}(A, B)}{A \times B}\right)$$

The 95% confidence intervals for the IC_{50} peptide concentration values were calculated as $IC_{50} \times 2$ (SEM) and then converted to a linear concentration scale for display in Tables 1 to 3.

Shell vial assay. We examined the activity of the peptides against chlamydial elementary bodies (EBs) in a recently described, standard shell vial assay (15). Briefly, coverslipped monolayers of McCoy cells grown in vials containing 1 ml of Eagle's minimal essential medium with 10% fetal bovine serum (EMEM/BS) were purchased from Bartels Diagnostics, Deerfield, Ill. The concentrated *C. trachomatis* EB seed stock was stored at −85°C in a mixture of 0.2 M sucrose, 0.004 M KH_2PO_4, 0.009 M Na_2HPO_4, and 0.004M glutamic acid (SPG medium). Prior to preincubation with peptides, dilutions of EB seed were made in SPG. Approximately 300 viable chlamydial EBs (30 μl of a 10^{-4} dilution of the seed) were mixed with 10 μl of peptide (various concentrations) in acidified water, in a total volume that was adjusted to 50 μl by adding SPG, and incubated for 2 h at room temperature. After carefully aspirating the EMEM/BS from the McCoy cells and replacing it with the *Chlamydia*/peptide mixture, the shell vials were centrifuged at 1,500 × *g* for 1 h at 20°C. After removing these inocula, the monolayers were washed twice with EMEM/BS containing 1 mg of cycloheximide (Bio Whittaker, Walkersville, Md.) per ml and incubated at 35°C for 48 h in 1 mL of the cycloheximide-containing EMEM/BS. Subsequent fixation with ethanol, staining with Microtrak fluorescein isothiocyanate-linked monoclonal antibody (Syva Company, San Jose, Calif.), and fluorescence microscopy were performed exactly as previously described (15).

Full-length protegrins. We recently reported that protegrin PG-1 protected cells from infection by *C. trachomatis* elementary bodies (15). In the present series of experiments wherein PG-1 was titrated against *C. trachomatis* in 15 experiments, its mean IC_{50} was ≈2.1 μg/ml. In two experiments wherein the concentrations of EBs were varied over a 25-fold range, the mean IC_{50} for PG-1 was 2.84 μg/ml under our standard conditions, 2.79 μg/ml when the EB concentration was fivefold higher, and 1.77 μg/ml when it was fivefold lower. As summarized in Table 1, which also shows their primary structures, PG-3 and PG-5 were about as effective as PG-1 in their activity against *C. trachomatis*. PG-4, notably less cationic than the other naturally occurring protegrins, was slightly less active than PG-1 ($P < 0.01$ at 3.16 μg/ml). Enantio-PG-1, an analog of PG-1 composed exclusively of D-amino acids, was indistinguishable from normal PG-1 in activity, as was a version of PG-1 whose C-terminal residue was not amidated.

Truncated protegrins. PG-2, a naturally occurring protegrin, lacks the C-terminal Gly-Agr residues present in full-length porcine protegrins. To determine if PG-1 could be appreciably shortened without losing activity against *C. trachomatis* EB, we synthesized the peptides shown in Table 2. PG-1 variants lacking residues 17 and 18 (PC-13) or 16 through 18 (PC-45) were less effective than the parent compound when tested at 3.16 μg/ml ($P < 0.002$). However, these compounds retained approximately 40 to 50% of the antichlamydial potency of the parent compound, indicating that modest C-terminal truncations could be accommodated. Antichlamydial activity also survived moderate N-terminal deletions, since a 14-mer that lacked residues 1 to 4 (PC-11) was as active as PC-13 and PC-45. However, deeper N-terminal deletions were deleterious, since PC-37 (which lacked residues 1 to 5) retained only about 10% of the activity of PG-1. Table 2 also indicates that

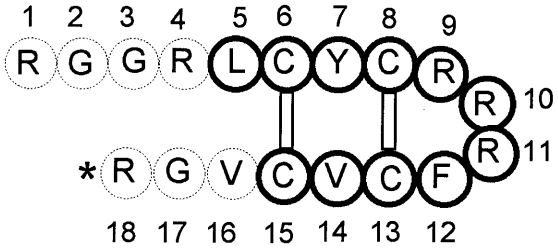

FIG. 1. Primary structure of protegrin PG-1. Residues 5 to 15, which are essential for the potent inactivation of *C. trachomatis* elementary bodies, are heavily outlined. Residues that can be deleted without substantially (>50%) reducing residual anti-chlamydial activity are surrounded by dashed circles. The cystine disulfide bonds are indicated by open bars. *, C-terminal amidation.

TABLE 3. Inactivation of *C. trachomatis* by disulfide variants of PG-1[a]

Peptide	Sequence[b]	No. of residues	No. of expts	IC_{50} (μg/ml) (95% C.I.)	Relative potency	100 μg/ml	31.6 μg/ml	10.0 μg/ml
PG-1	RGGRLCYCRRRFCVCVGR*	18	15	2.1 (1.76–2.40)	1.00	100 (0.0)	98.5 (0.48)	88.5 (1.34)
PC-8	RGGRL**A**Y**A**RRRF**A**V**A**VGR*	18	3	180.0[c]	0.01	39.4 (c) (9.3)	21.7 (c) (1.2)	13.4 (c) (0.8)
PC-9	RGGRL**A**YCRRRFCV**A**VGR*	18	3	10.8 (4.54–25.9)	0.20	91.3 (c) (3.8)	64.7 (c) (7.6)	48.8 (c) (6.4)
PC-10	RGGRLCYARRRFAVCVGR*	18	3	10.9 (8.2–14.4)	0.20	99.6 (a) (0.35)	86.0 (a) (5.5)	47.2 (c) (5.1)
PC-18	----LCY**A**RRRF**A**VCV--*	12	2	32.4 (22.7–46.3)	0.07	73.2 (c) (1.0)	49.5 (c) (3.8)	38.9 (c) (B)[d]
PC-19	----**R**CY**A**RRRF**A**VC**R**--*	12	2	37.8 (21.1–67.6)	0.06	80.7 (c) (2.4)	44.4 (c) (10.9)	39.4 (c) (B)
PC-20	----L**A**YCRRRFCV**A**V--*	12	2	93.9 (30.7–287)	0.02	50.6 (c) (5.7)	39.6 (c) (0.75)	28.3 (c) (B)
PC-21	----**RA**YCRRRFCV**AR**--*	12	2	61.2 (34.6–108.1)	0.03	64.3 (c) (2.1)	30.8 (c) (19.2)	24.2 (a) (B)

[a] See Tables 1 and 2 for more information.
[b] The primary sequences of PG-1 and the full-length and truncated disulfide variants are shown in standard single-letter code, with the disulfide bonds indicated. Residues that differ from the corresponding ones in PG-1 are shown in bold typeface.
[c] The IC_{50} value for PC-8 was extrapolated from the data shown in Fig. 2. For this reason, a 95% Confidence Interval was not calculated for this peptide.
[d] (B) indicates that this concentration was tested in only one of the two experiments.

biterminal truncations substantially diminished antichlamydial efficacy, as evidenced by the poor performance of PC-17, a 12-mer that lacked residues 1 to 4 and 17 and 18, and PC-73, a 10-mer that lacked residues 1 to 5 and 16 to 18. Overall, these data indicate that the ability of protegrins to inactivate *C. trachomatis* resides principally in residues 5 to 15 and that modestly truncated (14-mer and 15-mer) protegrin variants can retain substantial antimicrobial activity.

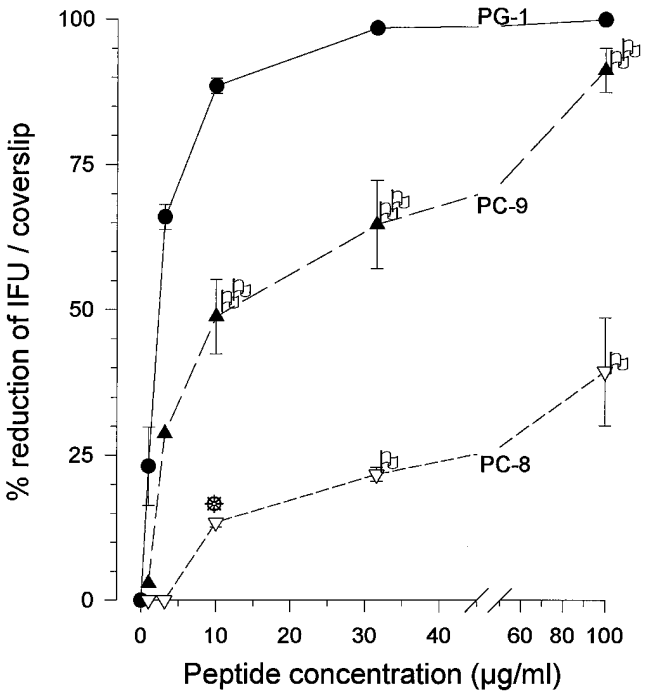

FIG. 2. Effect of disulfide bonds on activity against *C. trachomatis*. The ability of PG-1 and two full-length (18-mer) disulfide protegrin variants is shown. Datum points on each curve were compared, by Student's *t* test, with the corresponding datum points on the curve above them (i.e., PC-8 was compared to PC-9 and PC-9 was compared to PG-1). ▲, $P < 0.001$; ▽, $P < 0.01$; ❁, $P < 0.05$.

Disulfide variants. Naturally occurring protegrins, such as PG-1, contain two intramolecular disulfide bonds that connect Cys_6 to Cys_{15} and Cys_8 to Cys_{13} (Fig. 1). To determine the contribution of this feature to activity against *C. trachomatis*, we prepared several full-length and truncated congeners (Table 3) that lacked one or both cystine disulfides by virtue of paired Cys→Ala replacements. PC-8 ($ala_{6,8,13,15}$-PG-1), a congener of PG-1 that lacked both disulfide bonds, was approximately 1% as effective as regular PG-1. PC-9 and PC-10, 18-mers that contained but a single intramolecular S-S bond, were each about 20% as potent as normal PG-1 (Table 3 and Fig. 2). More truncated, 12-mer protegrin variants (PC 18, 19, 20, and 21) with single inramolecular disulfide bonds were somewhat more effective than PC-8 but were less active than the analogous 18-mers.

The above data indicated that full-length protegrins with 18 amino acids and two intramolecular disulfide bonds, such as PG-1, -3, and -5, showed optimal activity against *Chlamydia trachomatis* serovar L2 (L2/434/Bu). Truncation variants of PG-1, such as PC-11 and PC-45, that contained residues 5-15 of the parent compound (Fig. 1) manifested about half the potency of the parent molecule. Additional truncation or deleting one or both disulfides further reduced antichlamydial activity. We conclude that full-length or modestly truncated protegrins that retain residues 5 to 15 provide reasonable starting points for designing peptide and peptidomimetic molecules with enhanced antichlamydial potency for use in topical intravaginal preparations.

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