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Protegrin Structure and Activity against *Neisseria gonorrhoeae*

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Protegrin 1 (PG-1) is a broad-spectrum antimicrobial peptide that contains 18 amino acid residues (RG GRLCYCRRRFCVCVGR) and has two intramolecular cystine disulfide bonds. To determine the minimal structure responsible for protegrin-mediated activity against *Neisseria gonorrhoeae*, we synthesized 15 protegrin variants and tested them against two well-characterized gonococcal strains. The MICs of PG-1 were 0.61 μM (1.31 μg/ml) for the serum-sensitive strain F 62 and 0.98 μM (2.11 μg/ml) for the serum-resistant strain FA 19. Six amino acid residues (Arg_1, Gly_2, Gly_3, Arg_4, Gly_{17}, and Arg_{18}) and either disulfide bond could be deleted from PG-1 without impairing its potency against strain F 62. In contrast, only Gly_{17} and Arg_{18} could be removed without decreasing its activity against FA 19. Protegrin congener 64a (PC-64a; LTYCRRRFCVTV), a variant of PG-1 with 12 amino acid residues and one disulfide bond, displayed MICs of 0.45 μM (0.68 μg/ml) for strain F 62 and 1.37 μM (2.07 μg/ml) for strain FA 19, which approximated those of intact PG-1. Serum-sensitive *sac-1*$^+$ and *sac-3*$^+$ transformants of *N. gonorrhoeae* FA 19 and two FA 19 derivatives with truncated lipooligosaccharide structures were more susceptible to PG-1 and variants with altered disulfide structures. These data suggest that structurally simpler protegrin variants, such as PC-64a, could be used as topical microbicides for *N. gonorrhoeae*. They also suggest that the cystine-stabilized antiparallel β-sheet formed by PG-1 residues 5 to 16 is principally responsible for its activity against gonococci.

Protegrins are potent antimicrobial peptides that contain 16 to 18 amino acids that were originally purified from porcine leukocytes (10). They possess a β-sheet structure that is stabilized by two intramolecular disulfide bonds (4, 6). Low micromolar concentrations of several protegrins (protegrin 1 [PG-1], PG-2, PG-3, and PG-5) were recently reported to be highly active against *Neisseria gonorrhoeae*, including serum-resistant, serum-sensitive, and antibiotic-resistant strains (13). In contrast, defensins showed little activity against gonococci (13). Because protegrins also inactivated elementary bodies of *Chlamydia trachomatis* (17) and human immunodeficiency virus type 1 virions (16), they have considerable potential for use as topical agents to prevent sexually transmitted diseases.

This study was designed to determine the minimal protegrin structure needed for activity against *N. gonorrhoeae*. We tested a group of protegrin PG-1 congeners that included truncated forms and variants lacking one or both intramolecular disulfide bonds. We also investigated the mechanism of protegrin-mediated antigonococcal activity by testing transformants of a serum-resistant parental strain, FA 19, that possessed truncated lipooligosaccharide (LOS) structures (11) or mutations within the *sac-1* or *sac-3* gene (3, 14).

MATERIALS AND METHODS

Peptides. Synthetic protegrins and protegrin variants were used in this study. These peptides were prepared synthetically with 9-fluorenylmethoxycarbonyl chemistry at the Macromolecular Structure Facility of the University of Arizona and purified to homogeneity in our laboratory. After reduction with dithiothreitol, these peptides were purified by reverse-phase high-pressure liquid chromatography and dissolved in 0.1 M Tris at a peptide concentration of 1 mg/ml. Intramolecular disulfide bonds were formed by oxidizing the above peptide solutions at room temperature for 24 to 48 h. When unidisulfide protegrin variants were oxidized, 10% (vol/vol) dimethyl sulfoxide was added to enhance the efficiency of disulfide bond formation (15). All of the protegrins used in this study, including protegrin congener 73 (PC-73), contained three arginine residues in the β-turn region and were highly soluble (>1 mg/ml) in 0.01% acetic acid.

Bacteria. *N. gonorrhoeae* FA 19 (*sac-1 sac-3*; serum antibody and complement resistant [Sac^r]) and F 62 (*sac-1*$^+$ *sac-3*; serum sensitive [Sac^s]) were described previously (3). Strains FA 628 (*sac-1*$^+$ *sac-3*) and FA 899 (*sac-1 sac-3*$^+$) are Sac^s transformants of strain FA 19 (14). Strains FA 5101 and WS 1 are pyocin-resistant mutants of strain FA 19 (11) with the truncated LOS structures shown in Fig. 1.

Growth conditions. Bacteria were streaked on *N. gonorrhoeae* test medium plates (Clinical Standard Laboratories, Rancho Dominguez, Calif.), incubated overnight at 37°C in 5% CO_2–room air, and passaged daily. Nonopaque bacterial colonies from the plates were placed in 25 ml of GC broth (13) and incubated in a 37°C shaking water bath for 3 h to obtain mid-log-phase gonococci. An optical density at 620 nm of 0.1 corresponded to 10^8 CFU/ml.

Radial diffusion assays. Underlay and overlay gels were prepared as previously described in detail (13). Briefly, 4×10^6 CFU from an actively growing culture of *N. gonorrhoeae* was mixed with 10 ml of a molten (43°C) underlay gel solution and poured into 9- by 9-cm petri dishes to form a 1.23-mm gel composed of 1% agarose, 0.5% NaCl, 0.15% proteose peptone, 0.1% soluble starch, nutritional supplements, and phosphate buffer (13). A series of 3-mm-diameter wells was made to hold 5-μl aliquots of peptides that were dissolved and serially diluted in 0.01% acetic acid. Plates were incubated in a CO_2 incubator at 37°C for 3 h to allow peptides to diffuse into the underlays. Then a 10-ml overlay gel containing 1.5% proteose peptone, 1% agar, 0.5% NaCl, 0.1% soluble starch, nutritional supplements, and phosphate buffer was poured over the underlay (13). After overnight incubation, the diameters of the clear (colony-free) zones surrounding the wells were measured as previously described (13), and the MICs (micrograms per milliliter) were determined from the *x* intercepts of the curves.

RESULTS

Truncated protegrins. We previously reported that protegrins that had been purified from porcine leukocytes were highly active against *N. gonorrhoeae*, including strains that were serum resistant, serum sensitive, and antibiotic resistant (13). To determine if shorter protegrins retained activity against *N. gonorrhoeae*, we synthesized truncated protegrin congeners that lacked various C-terminal and/or N-terminal residues. Because the peptides varied considerably in mass, the MICs

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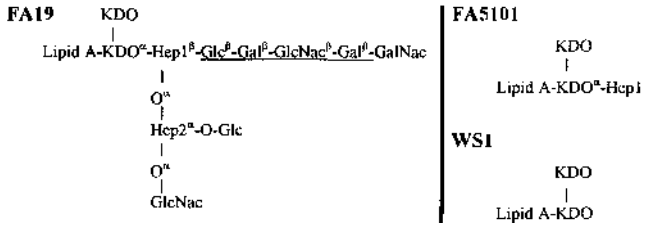

FIG. 1. LOS structures of *N. gonorrhoeae*. Abbreviations for sugar residues in the structure of LOS: KDO, 2-keto-3-deoxyoctonic acid; Hep, heptose; Glc, glucose; Gal, galactose; GlcNac, *N*-acetylglucosamine; GalNac, *N*-acetylgalactosamine. The lacto-*N*-neotetraose domain is underlined.

shown in Table 1 are expressed on a molar basis rather than in micrograms per milliliter.

PC-13, which lacked residues 17 and 18 (GR), showed enhanced molar potency against the serum-sensitive strain F 62 and intact activity against the serum-resistant strain FA 19. PC-11, a congener that lacked residues 1 to 4 (RGGR), retained its activity against F 62 but showed somewhat reduced activity against FA 19. PC-17, a 12-amino-acid peptide variant that lacked residues 1 to 4, 17, and 18, also retained its activity against F 62 and lost remarkably little of its effectiveness against FA 19. However, further truncations of PG-1 (e.g., PC-37, PC-45, PC-71, PC-72, and PC-73) were deleterious (Table 1), especially for activity against the serum-resistant strain FA 19.

Disulfide variants. Native protegrins contain two intramolecular disulfide bonds which connect residues Cys_6 to Cys_{15} and Cys_8 to Cys_{13}. To ascertain the contributions of these bonds to antigonococcal activity, we synthesized variants that lacked one or both of these disulfide bonds by virtue of paired alanine-for-cysteine substitutions. As shown in Table 1, PC-10, an 18-mer that contained a single Cys_6-to-Cys_{15} disulfide bond, was more potent on a molar basis than PG-1 against *N. gonorrhoeae* F 62 and FA 19. Although PC-9, its counterpart which contained only the Cys_8-to-Cys_{13} disulfide bond, retained full activity against F 62, it was approximately fourfold less potent against FA 19. The substantial contribution of intramolecular disulfide bonds for protegrin-mediated activity against *N. gonorrhoeae* was clearly shown by PC-8, a linearized variant of PG-1 that lacked both disulfide bonds. PC-8 showed a 16-fold reduction in activity against F 62 and a >200-fold reduction in activity against FA 19.

Because PC-17, a 12-mer variant of PG-1 with two disulfide bonds, had retained almost all of the potency of protegrins against *N. gonorrhoeae*, we also explored the antigonococcal activity of truncated unidisulfide variants. Although both PC-18 and PC-20, 12-mer versions of PC-10 and PC-9, respectively, retained over 50% of the molar potency of PG-1 against the serum-sensitive strain F 62, their activity against the serum-resistant strain FA 19 was markedly reduced. However, since PC-64 and PC-64a (variants of PC-18 and PC-20 peptides that contained threonines in place of the alanine residues) showed enhanced activity against F 62 and more modestly reduced activity against FA 19, the antigonococcal potential of unidisulfide 12-mers warrants further exploration.

Sac^s transformants of *N. gonorrhoeae* FA 19. We were intrigued that the serum-sensitive F 62 strain was more susceptible than the serum-resistant FA 19 strain to all of the protegrin variants shown in Table 1. Since susceptibility to lysis by serum antibody and complement is governed, in large part, by the organism's *sac* loci, we compared the susceptibilities of strains FA 628 (a *sac-1*$^+$ transformant of FA 19) and FA 899 (a *sac-3*$^+$ transformant of FA 19) to that of their isogenic parental strain, FA 19. As shown in Table 2, both transformants were significantly more susceptible than the parental strain to PG-1 and to each protegrin disulfide variant. Because the masses of PC-8, PC-9, and PC-10 were close to that of PG-1, the MICs in Table 2 are expressed in micrograms per milliliter.

TABLE 1. Susceptibility of *N. gonorrhoeae* to protegrins[a]

Peptide	Sequence	No. of residues	M_r	Mean MIC (μM) ± SEM	
				F 62	FA 19
PG-1	RGGRLCYCRRRFCVCVGR*	18	2,155	0.61 ± 0.03	0.98 ± 0.04
Truncated peptides					
PC-13	RGGRLCYCRRRFCVCV--*	16	1,943	0.34 ± 0.07 ••• ⇑	1.14 ± 0.26 NS
PC-45	RGGRLCYCRRRFCVC---*	15	1,843	0.58 ± 0.14 NS	4.11 ± 1.12 •••
PC-11	----LCYCRRRFCVCVGR*	14	1,730	0.75 ± 0.10 NS	1.72 ± 0.07 •••
PC-17	----LCYCRRRFCVCV--*	12	1,517	0.64 ± 0.15 NS	1.34 ± 0.15 ••
PC-37	-----CYCRRRFCVCVGR*	13	1,616	2.70 ± 0.29 •••	6.56 ± 0.40 •••
PC-71	-----CYCRRRFCVCV--*	11	1,403	3.20 ± 0.26 •••	10.0 ± 0.90 •••
PC-72	----LCYCRRRFCVC---*	11	1,417	1.70 ± 0.15 •••	9.51 ± 0.28 •••
PC-73	-----CYCRRRFCVC---*	10	1,304	22.9 ± 1.10 •••	79.9 ± 5.77 •••
Disulfide variants					
PC-10	RGGRLC**Y**A**R**RRF**A**VCVGR*	18	2,095	0.41 ± 0.04 ••• ⇑	0.79 ± 0.03 •• ⇑
PC-9	RGGRLA**Y**CRRRFCV**A**VGR*	18	2,095	0.63 ± 0.06 NS	4.15 ± 0.88 •••
PC-8	RGGRLA**Y**A**R**RRF**A**V**A**VGR*	18	2,032	10.0 ± 1.60 •••	223.7 ± 18.7 •••
PC-64	----LC**Y**T**R**RRF**T**VCV--*	12	1,514	0.48 ± 0.03 • ⇑	2.99 ± 0.19 •••
PC-64a	----L**T**YCRRRFCV**T**V--*	12	1,514	0.45 ± 0.06 • ⇑	1.37 ± 0.08 •••
PC-18	----LCYA**R**RRFAVCV--*	12	1,455	1.10 ± 0.37 •	7.65 ± 1.28 •••
PC-20	----LAYCRRRFCVAV--*	12	1,455	1.10 ± 0.24 ••	18.2 ± 3.36 •••

[a] PG-1 and its congeners are grouped in truncation and disulfide series. Their primary sequences are shown in standard single-letter code. Asterisks denote amidated C-terminal peptides. Boldface letters show amino acids that differ from PG-1. Peptides were tested four times except for PG-1 (n = 13), PC-8 (n = 12), PC-13 (n = 6), and PC-9 and PC-10 (n = 5). The activity of PG-1 was compared with those of its variants by Student's t test. •••, $P \leq 0.001$; ••, $P \leq 0.01$; •, $P \leq 0.05$; NS, not significant; ⇑, the peptide manifested greater potency than PG-1. Student's t test was also used to compare the susceptibilities of strains F 62 and FA 19 to the protegrin-like peptides listed above. With each peptide, strain F 62 was significantly more susceptible ($P \leq 0.05$).

TABLE 2. Effects of *sac* genes and LOS structure of *N. gonorrhoeae* on susceptibility to protegrins[a]

Protegrin	Mean MIC (μM) ± SEM (n = 5)				
	FA 19	FA 628 (*sac-1*$^+$)	FA 899 (*sac-3*$^+$)	FA 5101	WS 1
PG-1	1.03 ± 0.05	0.74 ± 0.03 •••	0.71 ± 0.02 •••	0.70 ± 0.04 •••	0.64 ± 0.06 •••
PC-10	0.79 ± 0.03	0.61 ± 0.02 ••	0.55 ± 0.06 ••	0.49 ± 0.06 ••	0.53 ± 0.05 ••
PC-9	4.15 ± 0.88	1.53 ± 0.24 •	1.38 ± 0.30 •	0.94 ± 0.23 ••	1.05 ± 0.22 ••
PC-8	196.6 ± 9.75	72.9 ± 3.86 •••	107.0 ± 14.2 •••	12.6 ± 2.63 •••	16.3 ± 3.61 •••

[a] FA 19 is the wild-type serum-resistant parental strain. FA 628 and FA 899 are isogenic to FA 19 except for their *sac* loci. FA 5101 and WS 1 are transformants of FA 19 with truncated LOS structures. Statistical significance was determined by Student's t test. The susceptibilities of FA 19 and its transformants to each peptide were compared. •••, $P \leq 0.001$; ••, $P \leq 0.01$; •, $P \leq 0.05$. The molecular weights of the peptides are as follows: PG-1, 2,155; PC-8, 2,032.4; and PC-9 and PC-10, 2,094.5.

Effect of LOS truncation. We used two other isogenic mutants of strain FA 19 to ascertain if LOS structure also affected the susceptibility of *N. gonorrhoeae* to protegrins: FA 5101, whose LOS contains lipid A (2-keto-3-deoxyoctonic acid [KDO])$_2$-heptose; and WS 1, whose LOS has only lipid A-(KDO)$_2$. As shown in Table 2, the LOS-truncated strains were considerably more susceptible to PC-8 ($P \leq 0.001$) and PC-9 ($P < 0.01$) than was the parental strain FA 19. LOS truncations also enhanced gonococcal susceptibility to PG-1 and PC-10 ($P < 0.01$), although these changes were of smaller magnitude.

DISCUSSION

Protegrins are unusually potent, broad-spectrum antimicrobial peptides that were originally purified from porcine leukocytes. Like the better-known defensins produced by human neutrophils (5), small intestinal Paneth cells (8, 9, 12), and genitourinary tract cells (2), protegrins have a well-defined structure with a substantial element of antiparallel β-sheet structure (1, 4). However, protegrins are considerably smaller than defensins (16 to 18 residues versus 29 to 35 residues) and contain only two rather than three intramolecular disulfide bonds.

In PG-1, the side chains of the underlined residues leucine 5, tyrosine 7, phenylalanine 12, and valines 14 and 16 (RGGRL CYCRRRFCVCVGR*) extend above the plane of the β-sheet, to form a well-ordered apolar cluster (4). Arginines 9, 10, and 11 participate in a β-turn which may move with a hinge motion. PC-17 is a truncated variant of PG-1 that lacks residues 1 to 4, 17, and 18 of its holopeptide parent, PG-1. Remarkably, PC-17 retained almost all of the antigonococcal activity of the parent molecule, suggesting that the structurally constrained and amphipathic central β-sheet portion of PG-1 is its critical antimicrobial component.

Our studies with one-disulfide (PC-9 and PC-10) and no-disulfide (PC-8) variants of PG-1 provided further evidence of the importance of the intramolecular disulfide bond(s) for antigonococcal activity. Whereas PC-8 was only about 1% as potent as PG-1 against *N. gonorrhoeae* FA 19 or its *sac-1*$^+$ or *sac-3*$^+$ isogenic transformant, PC-10 was actually a little more active than PG-1 against FA 19 ($P < 0.01$), FA 628 ($P < 0.01$), and FA 899 ($P < 0.05$). Although PC-9 was less potent against all three strains than PG-1 ($P < 0.001$), it was considerably more active than PC-8 ($P < 0.001$). These findings are consistent with evidence obtained in studies with liposome models that at least one intramolecular disulfide bond is needed for protegrins to retain antiparallel β-sheet conformation in membrane-mimetic environments, especially in the presence of physiological (extracellular) NaCl concentrations (7).

Our data also indicate that the LOS structure of *N. gonorrhoeae* can influence its susceptibility to protegrin-like antimicrobial peptides. *N. gonorrhoeae* mutants whose LOS structures were abbreviated to lipid A-(KDO)$_2$-heptose or lipid A-(KDO)$_2$ were inhibited by 10-fold-lower concentrations of PC-8 than the parental strain FA 19, and they were inhibited by about 4-fold-lower concentrations of PC-9. PG-1 binds to the lipid A moiety of *Escherichia coli* lipopolysaccharide with an affinity similar to that of polymyxin B (unpublished observations), but its interactions with gonococcal LOS remain to be examined.

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