Purification, Primary Structure, and Antimicrobial Activities of a Guinea Pig Neutrophil Defensin†

MICHAEL E. SELSTED[1,2]* AND SYLVIA S. L. HARWIG[1]

Departments of Medicine[1] and Pathology,[2] University of California Los Angeles School of Medicine, Los Angeles, California 90024

A broad-spectrum antimicrobial peptide present in guinea pig neutrophils was isolated, characterized biochemically, and assessed for microbicidal range and potency in vitro. The guinea pig neutrophil peptide (GPNP) was purified to homogeneity from a granule-rich subcellular fraction of peritoneal exudate neutrophils by gel filtration and reversed-phase high-performance liquid chromatography. GPNP was microbicidal for selected bacterial, fungal, and viral test organisms at concentrations in the microgram per milliliter range. Composition and primary structure analyses revealed that GPNP was homologous to a recently characterized family of antimicrobial peptides, termed defensins, isolated from rabbit and human neutrophils. The entire amino acid sequence of GPNP was determined, revealing that 8 of 31 residues were among those invariant in six rabbit and three human defensin peptides. The conserved sequence included six disulfide-linked cysteine residues, a common structural feature of defensins. The sequence of GPNP also included three nonconservative substitutions in positions otherwise invariant in the human and rabbit peptides. Characterization of GPNP provides new insight into structural features which may be essential for the broad-spectrum antimicrobial activities of defensins.

Numerous antimicrobial compounds have been implicated as effector molecules in the reactive oxygen intermediate (ROI)-independent killing of microbes ingested by phagocytic leukocytes (for reviews, see references 5 and 19). In previous communications we described the purification, characterization, and biological properties of nine structurally homologous antimicrobial peptides (defensins) from rabbit or human polymorphonuclear leukocytes (PMN, neutrophils; 6, 13, 17, 20, 22, 24–26). These defensins are peptides of 29 to 34 amino acid residues. Each contained 11 conserved residues, 6 of which were half-cystines. Defensins constitute of 5 to 15% of the total cellular protein of neutrophils (unpublished data) and are likely to contribute to their ROI-independent microbicidal activity. The in vitro antimicrobial spectra of the peptides vary but include several gram-positive and gram-negative bacteria (6, 26), fungi (6, 17, 20, 25), and selected enveloped viruses (3, 6, 13).

In their pioneering studies of mammalian PMN, Zeya and Spitznagel described the partial purification and characterization of antibacterial cationic proteins isolated from the lysosomes of guinea pig neutrophils (29, 30). They demonstrated that potent bactericidal activity was associated with three electrophoretically resolved arginine-rich polypeptides (30), which they distinguished from histones or granule enzymes (29). Lehrer et al. later demonstrated that cationic polypeptides from guinea pig neutrophil granules were fungicidal for *Candida parapsilosis* (15) and *Cryptococcus neoformans* (14). To determine whether the previously described antimicrobial activities might be attributable to polypeptides with structures or functions similar to those of the rabbit and human defensins, we purified the guinea pig peritoneal neutrophil peptide (GPNP) and showed it to be a member of the defensin family. By correlating certain of its unique features with the structures and activities of the

previously characterized defensins, we identified two arginine residues which may be essential for the potency and broad-spectrum activity of certain defensins.

MATERIALS AND METHODS

Reagents and chemicals. High-performance-liquid-chromatography (HPLC)-grade water and acetonitrile were purchased from Fisher Scientific Co., Pittsburgh, Pa. Trifluoroacetic acid (TFA), phenyl isothiocyanate, N-ethylmorpholine, HCl, and carboxypeptidase Y were from Pierce Chemical Co., Rockford, Ill. Acrylamides were from Bethesda Research Laboratories, Inc., Bethesda, Md. Trypsin was purchased from Worthington Diagnostics, Freehold, N.J.

Procurement of peritoneal neutrophils. Exudate cells containing predominantly neutrophils (>92%) were obtained by peritoneal lavage of anesthetized adult guinea pigs 12 to 18 h after an intraperitoneal injection of 60 ml of sterile phosphate-buffered saline containing 0.2% sodium caseinate and 50 ng of *Escherichia coli* lipopolysaccharide (Sigma Chemical Co., St. Louis, Mo.) per ml. Cells from peritoneal washes (ca. 300 ml per animal) were pooled, pelleted by centrifugation at 300 × g, and suspended in a small volume of 0.9% NaCl. After removal of a sample for total and differential cell counts, the cells were repelleted and either extracted directly with acetic acid (see below) or frozen at −20°C.

Purification of GPNP. An acid extract of whole neutrophils was prepared by homogenizing 1×10^8 to 10×10^8 cells in 10 ml of ice-cold 10% acetic acid in a Potter-Elvehjem homogenizer (18). The homogenate was subsequently stirred for 16 h at 4°C. Acid-insoluble material was removed by centrifugation at 27,000 × g for 20 min, and the supernatant was lyophilized. Lyophilized cell extract was dissolved in 2 to 10 ml of 10% acetic acid and fractionated on a 4.0- by 140-cm Bio-Gel P-10 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 5% acetic acid. The column effluent was monitored at 280 nm, and GPNP-containing fractions were identified by their electrophoretic migration (see be-
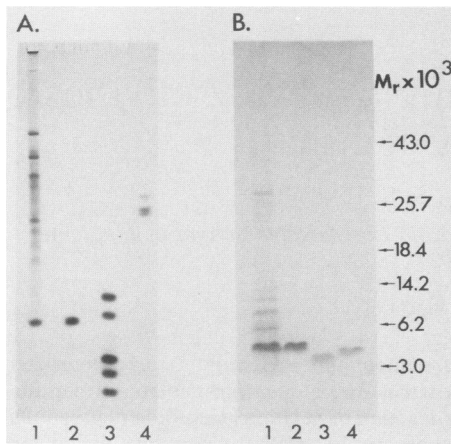

FIG. 1. PAGE of guinea pig, rabbit, and human defensins. (A) AU-PAGE. This Coomassie-stained 12.5% gel was loaded with 75 µg of guinea pig neutrophil acid extract (lane 1), 5 µg of purified GPNP (lane 2), 3 µg each of rabbit defensins NP-1 (most cathodal), NP-2, NP-3 (a plus b), NP-4, and NP-5 (least cathodal; lane 3), and 3 µg each of human defensins HNP-1 (most cathodal), HNP-2, and HNP-3 (least cathodal; lane 4). (B) SDS-PAGE. This Coomassie-stained gradient (10 to 30%) gel was loaded with 75 µg of guinea pig neutrophil acid extract (lane 1), 5 µg of purified GPNP (lane 2), 3 µg of human HNP-1 (lane 3), and 3 µg of rabbit NP-5 (lane 4). Each sample was boiled for 5 min in sample buffer containing 2-mercaptoethanol. Molecular weight standards run in parallel migrated to the positions indicated.

low) on acid-urea-containing polyacrylamide gels (AU-PAGE; 23). Fractions containing GPNP were pooled, lyophilized, and further purified by reversed-phase HPLC (RP-HPLC) on a C-18 column (1 by 25 cm; Vydac) by using an M45/680 binary solvent delivery system (Waters Associates, Inc., Milford, Mass.). Purity of the final product was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by AU-PAGE, and by analytical RP-HPLC.

Composition analysis. GPNP (100 µg) was reduced and carboxymethylated (8), desalted by RP-HPLC (6), and lyophilized in a centrifugal evaporator (Speed-Vac; Savant Instruments, Inc., Hicksville, N.Y.). Carboxymethylated peptide (50 µ) was hydrolyzed in vacuo in 5.7 N HCl for 24 to 72 h (24). Amino acids were quantitated by RP-HPLC on a C-18 column (PicoTag; Waters) after derivatization with phenyl isothiocyanate (24). Assay for reactive sulfhydryls was performed spectrophotometrically with Ellman reagent (9), and carbohydrate content was estimated by the phenol-sulfuric acid assay (4).

Trypsin digestion. Carboxymethylated GPNP (500 µg) was digested for 2 h at 37°C with 5 µg of trypsin in 250 µl of 0.1 M N-ethylmorpholine acetate, pH 8.20. Digestion was terminated by addition of 50 µl of acetic acid. The digestion products were purified by RP-HPLC on a C-18 column (4.6 by 250 mm; Vydac) as previously described (22). The composition of each purified fragment was determined by amino acid analysis as described above.

Sequence determination. Approximately 2 nmol of carboxymethylated GPNP was subjected to gas-phase Edman degradation on an Applied Biosystems, Inc., instrument. Phenylthiohydantoin (PTH) amino acids were identified by RP-HPLC on a Zorbax cyanopropyl column at 45°C (22). Additional analysis of the carboxyl-terminal residues of GPNP was performed by automated sequencing of the

carboxyl-terminal tryptic hexapeptide and by amino acid analysis of residues liberated by carboxypeptidase Y treatment (1).

Antimicrobial assays. The antimicrobial potency of GPNP was assessed by using selected bacterial, fungal, and viral test organisms. Detailed descriptions of the assay systems used have appeared previously as indicated below. Bactericidal assays were performed using group B streptococcus and *Pseudomonas aeruginosa* as test organisms (16). Fungicidal activity was assessed against yeast phase *Candida albicans* (25). Direct antiviral activity was tested using herpes simplex virus type 1 (HSV-1) McIntyre (3, 13).

RESULTS

Identification of GPNP. To ascertain whether guinea pig granulocytes contain defensinlike peptides, we fractionated acid extracts of neutrophil granules on preparative acid-urea gels as described previously (26). Inspection of electrophoretically resolved granule components revealed a prominent, rapidly migrating polypeptide (GPNP), as illustrated in Fig. 1, which also shows the migration of selected rabbit and human PMN defensins for comparison. Using a recently described eosin Y staining method (21), we purified a small amount of the rapidly migrating guinea pig neutrophil polypeptide from a preparative acid-urea gel as previously described (26) and found it to have potent antimicrobial activity against *Staphylococcus aureus* and *P. aeruginosa* (data not shown). The peptide had an apparent molecular weight of approximately 4,000 on gradient SDS-PAGE (Fig. 1B), further suggesting a similarity between GPNP and the previously characterized human and rabbit defensins.

Purification of GPNP. Milligram quantities of GPNP were obtained by subjecting acid-soluble cell extracts to sequential gel filtration and RP-HPLC purification steps. In early experiments GPNP was purified from the postnuclear (lysosome-rich) sediment of a neutrophil homogenate (15). We subsequently found that a higher yield of GPNP could be obtained by direct acid extraction of whole cells. Elution of the neutrophil extract from a Bio-Gel P-10 column was monitored at 280 nm, and GPNP-containing fractions were identified by their electrophoretic migration on acid-urea gels. GPNP eluted from the column under three A_{280}-absorbing peaks (Fig. 2). We have noted similar complex elution profiles of rabbit and human defensins when large amounts of material are resolved on this gel support. In the
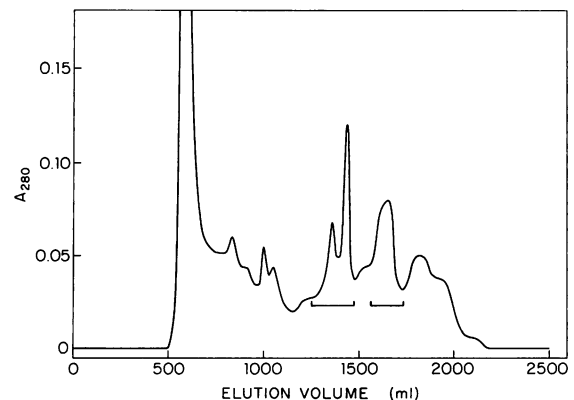

FIG. 2. P-10 gel filtration chromatography of GPNP. Crude acid extract from ca. 10^9 neutrophils was chromatographed at 40 ml/h. The eluant was monitored at 280 nm. ⌐──⌐, Fractions containing GPNP.
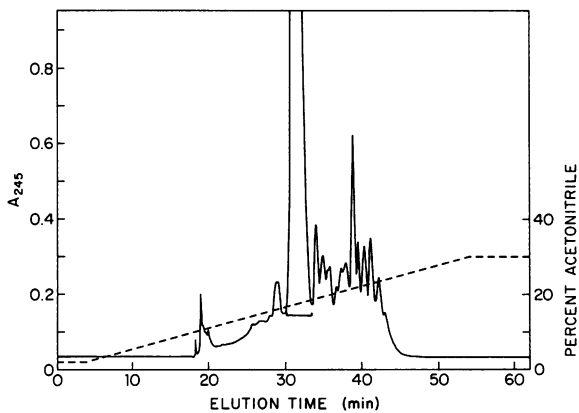
FIG. 3. RP-HPLC of GPNP. Lyophilized peptide from the P-10 gel filtration step was dissolved in 1.0 ml of 0.1% TFA and loaded onto a C-18 column (1.0 by 25 cm) equilibrated in 0.1% TFA in water. A linear gradient (----) of acetonitrile (0 to 30%) was developed for 50 min at 1.5 ml/min. The effluent was monitored at 245 nm. ⌐⌐, GPNP-containing fractions pooled.

subsequent RP-HPLC step (Fig. 3), a complex elution profile was obtained in which the major peak was GPNP. Final purification was achieved by recycling the GPNP-containing peak through an additional HPLC step, using the conditions described in the legend to Fig. 3. Purified peptide was homogeneous on RP-HPLC (Fig. 4), AU-PAGE (Fig. 1A), and SDS-PAGE (Fig. 1B).

Characterization of GPNP. (i) Composition analysis. Amino acid analysis (Table 1) revealed that GPNP, like the previously described rabbit and human defensins, was particularly rich in arginine and cysteine. GPNP did not contain methionine, tryptophan, serine, alanine, or lysine residues. Like the human defensins, but unlike the rabbit defensins, GPNP was rich in the aromatic residues tyrosine and phenylalanine. The minimum molecular weight by chemical analysis (M_r, 3,828) was consistent with the SDS-PAGE estimate of 4,000. Ellman reagent was unreactive with GPNP, indicating that the six half-cystine residues were present in three
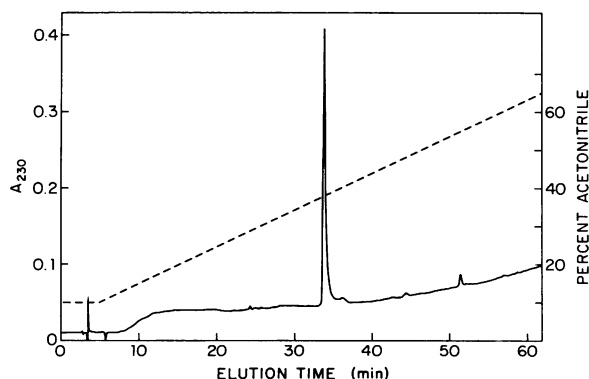
TABLE 1. Amino acid composition of GPNP[a]

Amino acid	No. of residues[b]
Arginine	6.92 (7)
Aspartic acid	0.90 (0)
Asparagine	0 (1)
Threonine	5.34 (5)
Glutamic	0.95 (0)
Glutamine	0 (1)
Proline	0.94 (1)
Glycine	1.05 (1)
Half-cystine[c]	6.06 (6)
Valine	1.03 (1)
Isoleucine	1.50 (2)
Leucine	1.32 (1)
Tyrosine	2.06 (2)
Phenylalanine	2.88 (3)

[a] Values determined from an analysis of a 72-h hydrolysate.
[b] Numbers in parentheses indicate residues as determined by sequence analysis.
[c] Determined as S-carboxymethylcysteine.

disulfide linkages. No carbohydrate was detectable by the phenol-sulfuric acid assay.

(ii) Sequence of GPNP. Carboxymethylated GPNP was sequenced on three occasions by gas-phase automated Edman degradation, with consistent results. The data shown in Table 2 are from sequencing run 3 and are representative. The sequence data were further confirmed by composition analysis of purified tryptic peptides (Table 3), each of which was unambiguously placed in the GPNP sequence (Fig. 5). Because of the relatively low yields obtained in sequencing the six carboxyl-terminal residues, the sequence of the carboxyl-terminal tryptic hexapeptide (T-7) was determined. As shown in Table 4, the sequence of T-7 was identical to that obtained by sequencing full-length GPNP (Table 2) and was consistent with the amino acid composition of T-7 (Table 3, Fig. 5). Digestion of carboxymethylated GPNP with carboxypeptidase Y resulted in the sequential release of S-carboxymethylcysteine, phenylalanine, threonine, and tyrosine, with kinetics and stoichiometry results consistent with the sequence of the carboxyl terminus (data not shown).

FIG. 4. Analytical RP-HPLC of GPNP. HPLC-purified GPNP (50 μg) was chromatographed on a C-18 column (4.6 by 250 mm). A 10 to 65% gradient (----) of acetonitrile was developed for 60 min at 1.0 ml/min. Heptafluorabutyric acid (0.13%) was used as an ion-pairing agent in both of the solvents (water and acetonitrile). Elution of GPNP was monitored spectrophotometrically at 230 nm. The small deflections in the base line at 45 and 52 min were also seen in peptide-free control runs.

TABLE 2. Gas-phase Edman degradation of GPNP[a]

Cycle no.	PTH amino acid	Yield[b]	Cycle no.	PTH amino acid	Yield
1	Arg	366	17	Leu	659
2	Arg	381	18	Gly	619
3	Cys	520	19	Thr	186
4	Ile	1,388	20	Cys	162
5	Cys	390	21	Ile	197
6	Thr	428	22	Phe	406
7	Thr	355	23	Gln	215
8	Arg	254	24	Asn	263
9	Thr	295	25	Arg	139
10	Cys	214	26	Val	99
11	Arg	149	27	Tyr	119
12	Phe	866	28	Thr	31
13	Pro	669	29	Phe	82
14	Tyr	670	30	Cys	53
15	Arg	274	31	Cys	38
16	Arg	318	32		

[a] Approximately 2 nmol of carboxymethylated GPNP was loaded on an Applied Biosystems 470A sequencer.
[b] Expressed as picomoles of identified PTH residue per degradation step.

TABLE 3. GPNP tryptic fragments[a]

Amino acid	No. of residues[b]						
	T-1	T-2	T-3	T-4	T-5	T-6	T-7
Arg	1.01 (1)	1.09 (1)	2.01 (2)	1.10 (1)	2.12 (2)	1.05 (1)	
Asp						0.86 (1)	
Thr	2.02 (2)	1.09 (1)	1.13 (1)			1.09 (1)	1.00 (1)
Glu						1.03 (1)	
Pro			0.96 (1)	0.92 (1)	0.95 (1)		
Gly						1.03 (1)	
Cys	1.89 (2)	0.81 (1)	0.83 (1)			0.92 (1)	2.01 (2)
Val							0.97 (1)
Ile	0.98 (1)					0.96 (1)	
Leu						1.06 (1)	
Tyr			0.85 (1)	1.03 (1)	0.95 (1)		0.98 (1)
Phe			1.05 (1)	0.94 (1)	0.98 (1)	1.00 (1)	1.04 (1)

[a] HPLC-purified carboxymethylated GPNP was digested with 1% (wt/wt) tosyl phenylalanyl chloromethyl ketone trypsin for 2 h at 37°C. Tryptic fragments (T) were purified by RP-HPLC. Samples were hydrolyzed for 24 h at 110°C and analyzed as phenylthiocarbamyl amino acids.

[b] See Table 1, footnote b.

Comparison of the primary structure of GPNP with those of rabbit and human defensins is shown in Fig. 6. Within the 10 sequences there exists an invariant core sequence of eight nonconsecutive residues which includes all six of the half-cystine residues. The residues conserved in all 10 peptides are three fewer than those common to the rabbit and human defensins, in which 11 residues are shared (7). Overall, human defensins are identical to GPNP in 14 of 31 (45.2%) of its residue positions, whereas NP-3b, the most homologous of the rabbit defensins, is identical in 11 of 31 (35.5%) of its residues.

(iii) **Antimicrobial activity of GPNP.** Preliminary experiments indicated that GPNP possessed in vitro microbicidal activity against several bacterial species. The data in Fig. 7A demonstrate the potency of GPNP against a gram-negative organism, *P. aeruginosa*. The bacterial inoculum was exposed to increasing concentrations of peptide for 20 min at 37°C. Note that ≥90% of the initial culture was killed by GPNP at concentrations as low as 20 μg/ml. Comparable sensitivity was shown after 20 min when we tested streptococcus (Fig. 7B), and substantially greater effect was evident when the incubation time was increased to 120 min. As expected, GPNP showed fungicidal activity against yeast-phase *C. albicans* (Fig. 7C). Exposure to 50 μg of GPNP per ml for 20 min resulted in a 99.9% decrease in yeast viability.

As we have previously noted with certain rabbit and human defensins (3, 13), GPNP directly neutralized HSV-1. Virucidal activity was dose dependent, as indicated by the data in Fig. 7D. Greater than 99.9% of the input viral titer was inactivated within 1 h by 75 μg of GPNP per ml at 37°C.

DISCUSSION

Recent evidence suggests that ROI-independent antimicrobial mechanisms play an important role in phagocyte-

mediated killing of potentially pathogenic microorganisms (5, 10–12, 19, 27). Lysosomal constituents, including lysozyme, lactoferrin, bactericidal permeability-increasing protein, and chymotrypsinlike cationic proteins, have been implicated as effector molecules in the killing of microorganisms by phagocytes independent of ROIs (5, 19). The recent molecular and biological characterization of defensins from rabbit and human neutrophils has defined a new class of natural antimicrobial compounds within the PMN.

The structural and functional attributes of GPNP clearly establish its identity as a newly characterized member of the defensin peptide family. Its antibacterial and antifungal activities are similar to those of the most active of the rabbit defensins (NP-1 and NP-2; 25, 26), and it has prominent antiviral activity against HSV-1.

Maximal alignment of the defensin sequences reveals that they each contain a conserved infrastructure made up of eight invariant residues (Fig. 6). Six of the conserved amino acids are half-cystines which constrain and stabilize the fold of the polypeptide backbone. Arginine 15 and glycine 18 are the other two conserved amino acids. Comparison of the human and rabbit defensins had previously suggested that the conserved defensin infrastructure was made up of 11 residues (Fig. 6). The finding that the sequence of GPNP varies from this consensus structure, as revealed by the three nonconservative substitutions at positions 6, 14, and 24, suggests that the essential structural residues in the defensins comprise relatively few amino acids.

Futher comparison reveals that GPNP resembles the six rabbit peptides in its amino-terminal sequence (X-X-cysteine) but mimics the human defensin sequence at its carboxyl terminus, i.e., cysteine is the terminal residue. The
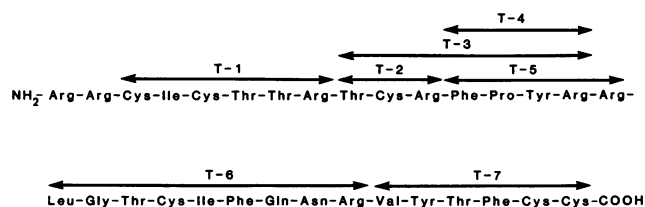

FIG. 5. Amino acid sequence of GPNP. The sequence of GPNP is shown, with the tryptic fragments (Table 3) indicated by the double-headed arrows.

TABLE 4. Gas-phase Edman degradation of GPNP tryptic peptide T-7[a]

Cycle no.	PTH amino acid	Yield
1	Val	7,405
2	Tyr	5,862
3	Thr	5,433
4	Phe	4,273
5	Cys	2,191
6	Cys	1,550
7		
8		

[a] An 8-nmol sample of tryptic fragment T-7 was sequenced on an Applied Biosystems instrument as described in the text.
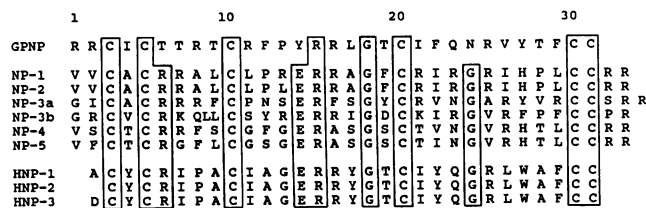
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          1               10              20                  30
GPNP   R R[C]I[C]T T R T[C]R F P Y[R R L[G]T[C]I F Q N R V Y T F[C C]

NP-1   V V C A C R R A L C L P R E R R A G F C R I R G R I H P L C C R R
NP-2   V V C A C R R A L C L P L E R R A G F C R I R G R I H P L C C R R R
NP-3a  G I C A C R R R F C P N S E R F S G Y C R V N G A R Y V R C C S R R
NP-3b  G R C V C R K QLL C S Y R E R R I G D C K I R G V R F P F C C P R
NP-4   V S C T C R R F S C G F G E R A S G S C T V N G V R H T L C C R R
NP-5   V P C T C R G F L C G S G E R A S G S C T I N G V R H T L C C R R

HNP-1  A C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
HNP-2    C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
HNP-3  D C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
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FIG. 6. Primary structures of antimicrobial peptides from guinea pig, rabbit, and human neutrophils. The amino acid sequences of GPNP and of homologous peptides from rabbit (NP 1 through 5) and human (HNP 1 through 3) neutrophils were maximally aligned. Conserved residues are bracketed. Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

cationicity of GPNP is intermediate among the defensin peptides. It migrates in AU-PAGE (Fig. 1A) at the expected position (between NP-3 [a and b] and NP-4) given its net charge (+7 at pH 2.5) and molecular size.

As noted previously, there exists among the members of the defensin family a significant natural diversity of structure and in vitro microbicidal spectrum and potency (6, 13, 17, 20, 22, 26). In addition to establishing the prevalence of defensins as major constituents of neutrophil granules in mammalian species, we have sought to discover new defensins to exploit their naturally occurring diversity in the study of structure-function relationships. By correlating unique biological activities with specific structural features, it may be possible to identify residues or peptide sequences essential for antimicrobial function. As an example, we have noted that GPNP, rabbit NP-1 and NP-2, and the three human defensins possess potent antiviral activity. These six peptides are distinguished from the nonvirucidal defensins by their arginine residues at positions 16 and 25 (Fig. 6). We are presently evaluating the effect of modification or substitution of these two residues on antiviral activity.

Defensins are major constituents of the neutrophil. By using quantitative staining of Coomassie-stained acid-urea gels (7), we have estimated that the cellular content of defensins in rabbit and human PMN is approximately 15 and 5%, respectively, of the total cellular protein. Given the short life-span of the neutrophil, it is apparent that the defensins represent a major biosynthetic investment by the host organism. The finding that the guinea pig neutrophil contains a member of the defensin family confirms the presence of these peptides in PMN from all species we have examined. This prevalence lends further support to their importance as effectors of phagocyte-mediated host defense.

The structural homology shared by the 10 defensins (Fig. 6) suggests that these peptides have arisen from a common ancestral gene. That each defensin peptide contains the conserved cystine framework suggests that their three-dimensional structures are likely to be similar. Ongoing crystallographic analyses of representative members of the defensin peptides will more directly address this question (28).

All of the defensins are acid-stable and relatively protease-resistant polypeptides that resemble other small, highly cross-linked polypeptides such as the protease inhibitors (2) and toxins from sea anemones and bee, elapid snake, and scorpion venom (2). Like the defensins, many of the toxins are quite stable on exposure to acid and elevated temperature and are inactivated by reduction and alkylation of their
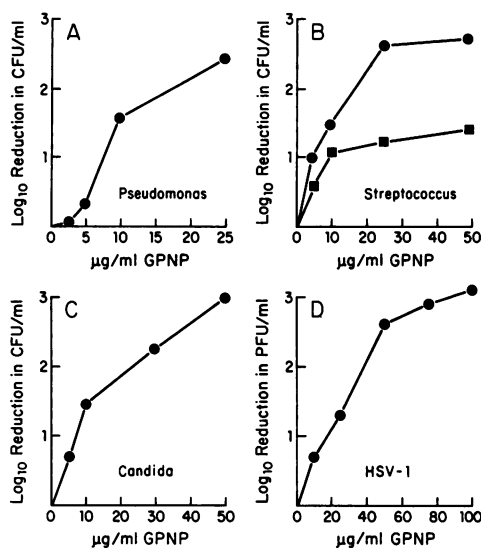

FIG. 7. Antimicrobial activity of GPNP. (A) Bactericidal activity of GPNP against *P. aeruginosa* PAO579. Log-phase organisms (2 × 10^5/ml) were incubated with increasing concentrations of GPNP in 10 mM sodium phosphate buffer, pH 7.4, for 20 min at 37°C. Antibacterial activity is expressed as a reduction in viable cells relative to that of the control. (B) Bactericidal activity of GPNP against group B streptococcus type 1a. Streptococcus was incubated with GPNP at the concentrations indicated in 10 mM phosphate buffer for 20 (■) or 120 (●) min. Survival data are expressed as described for panel A. (C) Fungicidal activity of GPNP against *C. albicans*. Yeast-phase *C. albicans* (2 × 10^5/ml) was incubated for 20 min with varied concentrations of GPNP. Killing is expressed as described for panels A and B. (D) Virucidal activity of GPNP against HSV-1. HSV-1 was grown in Vero cells and incubated with various concentrations of GPNP at a virus concentration of ca. 3 × 10^7 PFU/ml. After 1 h of incubation in phosphate-buffered saline at 37°C, the mixture was diluted serially in Eagle minimal essential medium–20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid) buffer containing 2% fetal calf serum and plated on Vero cell monolayers. Virucidal activity was quantitated by enumerating PFU after 48 h of incubation.

disulfides. The stability of the defensins to temperature and low pH is presumably conferred by their dense intramolecular disulfide cross-linking.

At present, much remains to be learned about the mechanism(s) by which defensins inactivate various microbial targets. Research in this area should benefit from the ability to correlate structural features of these peptides with their diverse antimicrobial potencies and target cell ranges.

ACKNOWLEDGMENTS

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